Cover Letter

XXX

Form Approved. O.M.B. Nos. 2070-0012 and 2070-0038

U.S. ENVIRONMENTAL PROTECTION AGENCY			AGENCY USE ONLY
EPA	PREMANUFACTURE NOTICE FOR NEW CHEMICAL SUBSTANCES		Date of receipt: 03/13/2020
When completed, send this form to:	If sending by Courier: Office of Pollution Prevention and Toxics Document Control Office (7407M) US EPA, 1201 Constitution Ave NW WASHINGTON, D.C. 20460 Contact Numbers: 202-564-8930/8940	If sending by US Mail: Office of Pollution Prevention and Toxics Document Control Office (7407M) US EPA, 1200 Pennsylvania Ave NW WASHINGTON, D.C. 20460	Submission Report Number

Total Number of Pages	TS Number
20	AB80KB

GENERAL INSTRUCTIONS

- You must provide all information requested in this form to the extent that it is known to or reasonably ascertainable by you. Make reasonable estimates if you do not have actual data.
- Before you complete this form, you should read the "Instructions Manual for Premanufacture Notification" (the Instructions Manual is available from the Toxic Substances Control Act (TSCA) Information Service by calling 202-554-1404, or faxing 202-554-5603).
- If a fee has been remitted for this notice (40 CFR 700.45), indicate in the boxes above the TS fee identification number you have generated. Remember, your fee ID number must also appear on your corresponding fee remittance. For mailing address information see the Help instructions in the e-PMN tool.

Part I – GENERAL INFORMATION

You must provide the currently correct Chemical Abstracts (CA) Name of the new chemical substance, even if you claim the identity as confidential. You may authorize another person to submit chemical identity information for you, but your submission will not be complete and the review will not begin until EPA receives this information. A letter in support of your submission should reference your TS fee identification number. For all Section 5 Notice submissions (paper or electronic) you must submit an original notice including all test data; if you claimed any information as confidential, an original sanitized copy must also be submitted.

Part II – HUMAN EXPOSURE AND ENVIRONMENTAL RELEASE

If there are several manufacture, processing, or use operations to be described in Part II, sections A and B of this notice, reproduce the sections as needed.

Part III – LIST OF ATTACHMENTS

For paper submissions, attach additional sheets if there is not enough space to answer a question fully. Label each continuation sheet with the corresponding section heading. In Part III, list these attachments, any test data or other data and any optional information included in the notice.

OPTIONAL INFORMATION

You may include any information that you want EPA to consider in evaluating the new substance. On page 11 of this form, space has been provided for you to describe pollution prevention and recycling information you may have regarding the new substance. "Binding" boxes are included throughout this form for you to indicate your willingness to be bound to certain statements you make in this section, such as use, production volume, protective equipment . . . The intention is to reduce delays that routinely accompany the development of consent orders or Significant New Use Rules. Checking a "binding" box in a PMN does not by itself prohibit the submitter from later deviating from the information (except chemical identity) reported in the form; however, in the case of exemption applications (such as TMEA, LVE, LOREX) certain information provided in such notifications is binding on the submitter when the Agency approves the exemption application, especially if the production volume "binding" box is chosen in a LVE.

CONFIDENTIALITY CLAIMS

You may claim any information in this notice as confidential. To assert a claim on the form, mark (X) the confidential box next to the information that you claim as confidential. To assert a claim in an attachment, circle or bracket the information you claim as confidential. If you claim information in the notices as confidential, you must also provide a sanitized version of the notice, (including attachments). For additional instructions on claiming information as confidential, read the Instructions Manual.

TEST DATA AND OTHER DATA

You are required to submit all test data in your possession or control and to provide a description of all other data known to or reasonably ascertainable by you, if these data are related to the health and environmental effects on the manufacture, processing, distribution in commerce, use, or disposal of the new chemical substance. Standard literature citations may be submitted for data in the open scientific literature. Complete test data (written in English), not summaries of data, must be submitted if they do not appear in the open literature. You should clearly identify whether test data is on the substance or on an analog. Also, the chemical composition of the tested material should be characterized. Following are examples of test data and other data. Data should be submitted according to the requirements of §720.50 of the Premanufacture Notification Rule (40 CFR Part 720).

Test Data (Check Below any included in this notice)

[X]	Environmental fate data	[ ]	Other Data
[X]	Health effects data	[ ]	Risk Assessments
[ ]	Environmental effects data	[ ]	Structure/activity relationships
[X]	Physical/Chemical Properties (A physical and chemical properties worksheet is located on the last page of this form.)		
[ ]	Test data not in the possession or control of the submitter		

TYPE OF NOTICE (Check Only One)

- [X] **PMN** (Premanufacture Notice)
- [ ] **SNUN** (Significant New Use Notice)
- [ ] **TMEA** (Test Marketing Exemption Application)
- [ ] **LVE** (Low Volume Exemption) @ 40 CFR 723.50(c)(1)
- [ ] **LOREX** (Low Release/Low Exposure Exemption) @ 40 CFR 723.50(c)(2)
- [ ] **LVE** Modification
- [ ] **LOREX** Modification
- [ ] **Mock Submission**
- [ ] Mark (X) if pending Letter of Support

N — IS THIS A CONSOLIDATED PMN (Y/N)?

1 — # of chemicals or polymers (Prenotice Communication # required, enter # on p. 3).

[X] Mark (X) if any information in this notice is claimed as confidential.

The public reporting and recordkeeping burden for this collection of information is estimated to average 93 hours per response. Send comments on the Agency's need for this information, the accuracy of the provided burden estimates, and any suggested methods for minimizing respondent burden, including through the use of automated collection techniques to the Director, Collection Strategies Division, U.S. Environmental Protection Agency (2822T), 1200 Pennsylvania Ave., NW, Washington, D.C. 20460. Include the OMB control number in any correspondence. Do not send the completed EPA Form 7710-25 to this address.

CERTIFICATION -- A printed copy of this signature page, with original signature, must be submitted with CD or paper submission.

I hereby certify to the best of my knowledge and belief that all information entered on this form is complete and accurate. I further certify that, pursuant to 15 U.S.C. § 2613(c), for all claims for protection for any confidential information made with this submission, all information submitted to substantiate such claims is true and correct, and that it is true and correct that the person submitting the claim has:

(i) taken reasonable measures to protect the confidentiality of the information;

(ii) determined that the information is not required to be disclosed or otherwise made available to the public under any other Federal law

(iii) a reasonable basis to conclude that disclosure of the information is likely to cause substantial harm to the competitive position of the person; and

(iv) a reasonable basis to believe that the information is not readily discoverable through reverse engineering.

Any knowing and willful misrepresentation is subject to criminal penalty pursuant to 18 U.S.C. § 1001.

Additional Certification Statements:

If you are submitting a PMN, SNUN, LoREX, LVE, or TMEA, check the following Fees Certification statement that applies:

- ☐ The Company named in Part I, Section A is a "small business concern" as defined under 40 CFR 700.43 and will remit the fee as specified in 40 CFR 700.45(c).
- ☒ The Company named in Part I, Section A will remit the fee as specified in 40 CFR 700.45(c).
- ☐ This joint submission includes at least one Company which is a "small business concern" and at least one Company which is not a "small business concern," as defined under 40 CFR 700.43. The fee will be remitted with the joint submission. Any remaining balance due for this joint submission is to be paid by the secondary submitter(s).
- ☐ The company named in Part I, Section A is submitting a sustainable futures TME. The company has graduated from EPA's Sustainable Futures program and is therefore exempt from fees for this sustainable futures TME.

If you are submitting a **Low Volume Exemption (LVE)** application in accordance with 40 CFR 723.50(c)(1) or a **Low Release and Low Exposure Exemption (LoRex)** application in accordance with 40 CFR 723.50(c)(2), check the following certification statements:

- ☐ The manufacturer submitting this notice intends to manufacture or import the new chemical substance for commercial purposes, other than in small quantities solely for research and development, under the terms of 40 CFR 723.50.
- ☐ The manufacturer is familiar with the terms of this section and will comply with those terms; and
- ☐ The new chemical substance for which the notice is submitted meets all applicable exemption conditions.
- ☐ If this application is for an LVE in accordance with 40 CFR 723.50(c)(1), the manufacturer intends to commence manufacture of the exempted substance for commercial purposes within 1 year of the date of the expiration of the 30 day review period.

				Confidential
Signature and title of Authorized Official (Original Signature Required)	*XXX*	Date	XXX	☒

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Part I -- GENERAL INFORMATION

Section A – SUBMITTER IDENTIFICATION

Mark (X) the "Confidential" box next to any subsection you claim as confidential

1a.	Person Submitting Notice (in U.S.)					Confidential
Name of Authorized Official	(first) XXX		(last) XXX			X
Position	XXX					
Company	XXX					
Mailing Address (number & street)	XXX					
City		State		Postal Code	XXX	
email	XXX					

b.	Agent (if Applicable)					Confidential
Name of Authorized Official	(first)		(last)			☐
Position						
Company						
Mailing Address (number & street)						
City		State		Postal Code		
e-mail		Telephone (include area code)				

c.	Joint Submitter (if applicable)					Confidential
If you are submitting this notice as part of a joint submission, mark (X)					☐	☐
Name of Authorized Official	(first)		(last)			
Position						
Company						
Mailing Address (number & street)						
City		State		Postal Code		
e-mail		Telephone (include area code)				

2.	Technical Contact (in U.S.)					Confidential
Name of Authorized Official	(first) XXX		(last) XXX			X
Position	XXX					
Company	XXX					
Mailing Address (number & street)	XXX					
City	XXX	State	XXX	Postal Code	XXX	
e-mail	XXX	Telephone (include area code)		XXX		

			Mark (X) if none	Confidential
3.	If you have had a prenotice communication (PC) concerning this notice and EPA assigned a PC Number to the notice, enter the number.		X	☐
4.	If you previously submitted an exemption application for the chemical substance covered by this notice, enter the exemption number assigned by EPA. If you previously submitted a PMN for this substance enter the PMN number assigned by EPA (i.e. withdrawn or incomplete).		Mark (X) if none X	Confidential ☐
5.	If you have submitted a notice of Bona fide intent to manufacture or import for the chemical substance covered by this notice, enter the notice number assigned by EPA.		Mark (X) if none X	Confidential ☐

6.	Type of Notice – Mark (X)						
1.	Manufacture Only Binding Option	X ☐	2.	Import Only Binding Option	☐ ☐	3.	Both ☐

Part I – GENERAL INFORMATION -- Continued

Section B – CHEMICAL IDENTITY INFORMATION: You must provide a currently correct Chemical Abstracts (CA) name of the substance based on current CA index nomenclature rules and conventions.

Mark (X) the "Confidential" box next to any item you claim as confidential

Complete either item 1 (Class 1 or 2 substances) or 2 (Polymers) as appropriate. Complete all other items.

If another person will submit chemical identity information for you (for either Item 1 or 2), mark (X) the box at the right. Identify the name, company, and address of that person in a continuation sheet. ☐

1. Class 1 or 2 chemical substances (for definitions of class 1 and class 2 substances, see the Instructions Manual)	Class 1	Class 2	CBI
a. Class of substance - Mark (X)	☐	☐	X

b. Chemical name (Currently correct Chemical Abstracts (CA) Name that is consistent with TSCA Inventory listings for similar substances. For Class 1 substances a CA Index Name must be provided. For Class 2 substances either a CA Index Name or CA Preferred Name must be provided, which ever is appropriate based on current CA index nomenclature rules and conventions). X

XXX

CAS Registry Number (if a number already exists for the substance): XXX

c. Please identify which method you used to develop or obtain the specified chemical identity information reported in this notice: (check one).

Method 1 (CAS Inventory Expert Service - a copy of the Identification report obtained from the CAS Inventory Expert Services must be submitted as an attachment to this notice) X

IES Order Number: 439408-3

Method 2 (Other Source) ☐

Enter Attachment filename for Part I, Section B, 1. c.: Sanitized Document: 2 CAS-IES Report_Redacted.pdf X

d. Molecular formula: XXX X

e. For a class 1 substance, provide a complete and correct chemical structure diagram. For a class 2 substance, provide a correct representative or partial chemical structure diagram, as complete as can be known, if one can be reasonably ascertained. X

See Attachment (Sanitized Document: 1 Structure Diagram_Redacte...
)

Enter Attachment filename for Part I, Section B, 1. e. ☐

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For a class 2 substance - (1) List the immediate precursor substances with their respective CAS Registry Numbers. (2) Describe the nature of the reaction or process. (3) Indicate the range of composition and the typical composition (where appropriate).	Confidential

e. (1) List the immediate precursor substance names with their respective CAS Registry Numbers. ☐

Enter Attachment filename for Part I, Section B, 1. e. (1)		☐

e. (2) Describe the nature of the reaction or process. ☐

Enter Attachment filename for Part I, Section B, 1. e. (2)		☐

e. (3) Indicate the range of composition and the typical composition (where appropriate). ☐

Enter Attachment filename for Part I, Section B, 1. e. (3)		☐

Part I -- GENERAL INFORMATION -- Continued

Section B -- CHEMICAL IDENTITY INFORMATION -- Continued

3. Impurities
   (a) - Identify each impurity that may be reasonably anticipated to be present in the chemical substance as manufactured for commercial purpose. Provide the CAS Registry Number if available. If there are unidentified impurities, enter "unidentified."
   (b) - Estimate the maximum weight % of each impurity. If there are unidentified impurities, estimate their total weight %.

Impurity (a)	CAS Registry Number (a)	Maximum Percent % (b)	Confi-dential
XXX	XXX	XXX	X
XXX	XXX	XXX	X
XXX	XXX	XXX	X
XXX	XXX	XXX	X

Mark (X) this box if the data continues on the next page. ☐

Enter Attachment filename for Part I, Section B, 3. ☐

4. Synonyms - Enter any chemical synonyms for the new chemical identified in subsection 1 or 2.
XXX

☒

Enter Attachment filename for Part I, Section B, 4. ☐

5. Trade identification - List trade names for the new chemical substance identified in subsection 1 or 2.
XXX

☒

Enter Attachment filename for Part I, Section B, 5. ☐

6. Generic chemical name - If you claim chemical identify as confidential, you must provide a generic name for your substance that reveals the specific chemical identity of the new chemical substance to the maximum extent possible. Refer to the TSCA Chemical Substance Inventory, 1985 Edition, Appendix B for guidance on developing generic names.

Perfluorodioxaalkyl vinyl ether,

Enter Attachment filename for Part I, Section B, 6.

7. Byproducts - Describe any byproducts resulting from the manufacture, processing, use, or disposal of the new chemical substance. Provide the CAS Registry Number if available.

Byproduct (1)	CAS Registry Number (2)	Confi-dential

Mark (X) this box if the data continues on the next page. ☐

Part I -- GENERAL INFORMATION – Continued

Section B -- CHEMICAL IDENTITY INFORMATION -- Continued

2. Polymers (For a definition of polymer, see the Instructions Manual.)

Confidential

a. Indicate the number-average weight of the lowest molecular weight composition of the polymer you intend to manufacture. Indicate maximum weight percent of low molecular weight species (not including residual monomers, reactants, or solvents) below 500 and below 1,000 absolute molecular weight of that composition. ☐

Describe the methods of measurement or the basis for your estimates:

GPC ☐ | **Other** (Specify Below) ☐

Specify Other:

(i) lowest number average molecular weight:	(ii) maximum weight % below 500 molecular weight:	(iii) maximum weight % below 1000 molecular weight:

Enter Attachment filename for Part I, Section B, 2. a. ☐

b. You must make separate confidentiality claims for monomer or other reactant identity, composition information, and residual information. Mark (X) the "Confidential" box next to any item you claim as confidential

(1) - Provide the specific chemical name and CAS Registry Number (if a number exists) of each monomer or other reactant used in the manufacture of the polymer.
(2) - Mark (X) this column if entry in column (1) is confidential.
(3) - Indicate the typical weight percent of each monomer or other reactant in the polymer.
(4) - Choose "yes" from drop down menu if you want a monomer or other reactant used at two weight percent or less to be listed as part of the polymer description on the TSCA Chemical Substance Inventory.
(5) - Mark (X) this column if entries in columns (3) and (4) are confidential.
(6) - Indicate the maximum weight percent of each monomer or other reactant that may be present as a residual in the polymer as manufactured for commercial purposes.
(7) - Mark (X) this column if entry in column (6) is confidential.

Monomer or other reactant specific chemical name (**1**)	CBI (**2**)	Typical composition (**3**)	Include in identity (**4**)	CBI (**5**)	Max residual (**6**)	CBI (**7**)
CAS Registry Number (**1**)						
CAS Registry Number (**1**)						
CAS Registry Number (**1**)						
CAS Registry Number (**1**)						
CAS Registry Number (**1**)						

Mark (X) this box if the data continues on the next page. ☐

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c. Please identify which method you used to develop or obtain the specified chemical identity information reported in this notice (check one).				CBI
Method 1 (CAS Inventory Expert Service - a copy of the identification report obtained from CAS Inventory Expert Service must be submitted as an attachment to this notice) ☐	IES Order Number		**Method 2** (other source) ☐	
Enter Attachment filename for Part I, Section B, 2. c.				☐

d. The currently correct Chemical Abstracts (CA) name for the polymer that is consistent with TSCA Inventory listings for similar polymers.	☐

CAS Registry Number (if a number already exists for the substance)		

e. Provide a correct representative or partial chemical structure diagram, as complete as can be known, if one can be reasonably ascertained.	☐

Enter Attachment filename for Part I, Section B, 2. e.		☐

Part I -- GENERAL INFORMATION -- Continued

Section C -- PRODUCTION, IMPORT, AND USE INFORMATION:

The information on this page refers to consolidated chemical number(s): [X] **1** [ ] **2** [ ] **3** [ ] **4** [ ] **5** [ ] **6**

Mark (X) the "Confidential" box next to any item you claim as confidential.

1. Production volume -- Estimate the **maximum** production volume during the first 12 months of production. Also estimate the maximum production volume for any consecutive 12-month period during the first three years of production. Estimates should be on 100% new chemical substance basis. For a Low Volume Exemption application, if you choose to have your notice reviewed at a lower production volume than 10,000 kg/yr, specify the volume and mark (x) in the binding box. If granted, you are bound to this volume.

Maximum first 12-month production (kg/yr) (100% new chemical substance basis)	Maximum 12-month production (kg/yr) (100% new chemical substance basis)	Confidential	Binding Option Mark (X)
XXX	XXX	[X]	[ ]

Enter Attachment filename for Part I, Section C, 1.		CBI [ ]

2. Use Information -- You must make separate confidentiality claims for the description of the category of use, the percent of production volume devoted to each category, the formulation of the new substance, and other use information. Mark (X) the "Confidential" Box next to any item you claim as confidential.

a. (1) --Describe each intended category of use of the new chemical substance by function and application.
(2) --Mark (X) this column if entry column (1) is confidential business information (CBI).
(3) --Indicate your willingness to have the information provided in column (1) binding.
(4) --Estimate the percent of total production for the first three years devoted to each category of use.
(5) --Mark (X) this column if entry in column (4) is confidential business information (CBI).
(6) --Estimate the percent of the new substance as formulated in mixtures, suspensions, emulsions, solutions, or gels as manufactured for commercial purposes at sites under your control associated with each category of use.
(7) --Mark (X) this column if entry in column (6) is confidential business information (CBI).
(8) --Indicate % of product volume expected for the listed "use" sectors. Mark more than one box if appropriate. Mark (X) to indicate your willingness to have the use type provided in (8) binding.
(9) --Mark (X) this column if entry(ies) in column (8) is (are) confidential business information (CBI).

Category of use (**1**) (by function and application i.e. a dispersive dye for finishing polyester fibers)	CBI (**2**)	Binding Option Mark (X) (**3**)	Production % (**4**)	CBI (**5**)	% in Formulation (**6**)	CBI (**7**)	% of substance expected per use (**8**)					CBI (**9**)
							Site-limited	Consumer*	Industrial	Commercial	Binding Option	
XXX	X		XXX	X	XXX	X	XXX	XXX	XXX	XXX		X

* If you have identified a "consumer" use, please provide on a continuation sheet a detailed description of the use(s) of this chemical substance in consumer products. In addition include estimates of the concentration of the new chemical substance as expected in consumer products and describe the chemical reactions by which this substance loses its identity in the consumer product.

Mark (X) this box if the data continues on the next page. [ ]

b. Generic use description — If you claim any category of use description in subsection 2a as confidential, enter a generic description of that category. Read the Instruction Manual for examples of generic use descriptions.

Intermediate

Enter Attachment filename for Part I, Section C, 2. b.		CBI [ ]

3. Hazard Information -- Include in the notice a copy of reasonable facsimile of any hazard warning statement, label, material safety data sheet, or other information which will be provided to any person who is reasonably likely to be exposed to this substance regarding protective equipment or practices for the safe handing, transport, use, or disposal of the new substance. List in part III hazard information you include.

Mark (X) this box if you attach hazard information.	[X]	Binding Option Mark (X) [ ]

Part II-- HUMAN EXPOSURE AND ENVIRONMENTAL RELEASE

Section A -- INDUSTRIAL SITES CONTROLLED BY THE SUBMITTER

Mark (X) the "Confidential" box next to any item you claim as confidential

The information on pages 8 and 8a refer to consolidated chemical number(s): [X] **1** [ ] **2** [ ] **3** [ ] **4** [ ] **5** [ ] **6**

Complete section A for each type of manufacture, processing, or use operation involving the new chemical substance at industrial sites you control. Importers do not have to complete this section for operations outside the U.S.; however, you may still have reporting requirements if there are further industrial processing or use operations after import. You must describe these operations. See instructions manual

1. Operation description
   a. Identity -- Enter the identity of the site at which the operation will occur.

				Confi-dential
Name	XXX			
Site address (number and street)	XXX			[X]
City	XXX	County	XXX	
State	XXX	ZIP code	XXX	

If the same operation will occur at more than one site, enter the number of sites. Identify the additional sites on a continuation sheet, and if any of the sites have significantly different production rates or operations, include all the information requested in this section for those sites as attachments. →	XXX	[X]
Mark (X) this box if the data continues on the next page.	[ ]	

b. Type -- Mark (X) Manufacturing [ ] Processing [ ] Use [ ] — [X]

c. Amount and Duration -- Complete 1 or 2 as appropriate

				Confi-dential
1. Batch	Maximum kg/batch (100% new chemical substance)	Hours/batch	Batches/year	[ ]
2. Continuous	Maximum kg/day (100% new chemical substance)	Hours/day	Days/year	[X]
	XXX	XXX	XXX	

d. Process description — Mark (X) to indicate your willingness to have your process description binding. → [ ]

(1) Diagram the major unit operation steps and chemical conversions. Include interim storage and transport containers (specify- e.g. 5 gallon pails, 55 gallon drum, rail car, tank truck, etc.).
(2) Provide the identity, the approximate weight (by kg/day or kg/batch on a 100% new chemical substance basis), and entry point of all starting materials and feedstocks (including reactants, solvents, catalysts, etc.), and of all products, recycle streams, and wastes. Include cleaning chemicals (note frequency if not used daily or per batch.).
(3) Identify by number the points of release, including small or intermittent releases, to the environment of the new chemical substance. If releasing to two media at the same step, assign a second release number for the second medium.

[X]

XXX

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Diagram of the major unit operation steps.	Confidential
	X

See Attachment (Sanitized Document: 5 Process Diagram_Substance...
)

Enter Attachment filename for Part II, Section A, 1. d.	Sanitized Document: 5 Process Diagram_Substance... X

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Part II-- HUMAN EXPOSURE AND ENVIRONMENTAL RELEASE -- Continued

Section A -- INDUSTRIAL SITES CONTROLLED BY THE SUBMITTER -- Continued

The information on pages 9 and 9a refer to consolidated chemical number(s): [X] **1** [ ] **2** [ ] **3** [ ] **4** [ ] **5** [ ] **6**

2. Occupational Exposure -- You must make separate confidentiality claims for the description of worker activity, physical form of the new chemical substance, number of workers exposed, and duration of activity. Mark (X) the "Confidential" box next to any item you claim as confidential.

(1) -- Describe the activities (i.e. bag dumping, tote filling, unloading drums, sampling, cleaning, etc.) in which workers may be exposed to the substance.
(2) -- Mark (X) this column if entry in column (1) is confidential business information (CBI).
(3) -- Describe any protective equipment and engineering controls used to protect workers.
(4) and (6) -- Indicate your willingness to have the information provided in column (3) or (5) binding.
(5) -- Indicate the physical form(s) of the new chemical substance (e.g., solid: crystal, granule, powder, or dust) and % new chemical substance (if part of a mixture) at the time of exposure.
(7) -- Mark (X) this column if entries in columns (3) and (5) are confidential business information (CBI).
(8) -- Estimate the maximum number of workers involved in each activity for all sites combined.
(9) -- Mark (X) this column if entry in column (8) is confidential business information (CBI).
(10) and (11) -- Estimate the maximum duration of the activity for any worker in hours per day and days per year.
(12) -- Mark (X) this column if entries in columns (10) and (11) are confidential business information (CBI).

Worker activity (i.e., bag dumping, filling drums) (1)	CBI (2)	Protective Equipment/ Engineering Controls (3)	Binding Option Mark (X) (4)	Physical form(s) & % new substance (5)	Binding Option Mark (X) (6)	CBI (7)	# of Workers Exposed (8)	CBI (9)	Maximum Duration		CBI (12)
									Hrs/Day (10)	Days/Yr (11)	
XXX	X	XXX		XXX		X	XXX	X	XXX	XXX	X
XXX	X	XXX		XXX		X	XXX	X	XXX	XXX	X
XXX	X	XXX		XXX		X	XXX	X	XXX	XXX	X
XXX	X	XXX	X	XXX	X	X	XXX	X	XXX	XXX	X
XXX	X	XXX		XXX		X	XXX	X	XXX	XXX	X

Mark (X) this box if the data continues on the next page. [ ]

Enter Attachment filename for Part II, Section A on the bottom of page 9a. [ ]

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3. Environmental Release and Disposal -- You must make separate confidentiality claims for the release number and the amount of the new chemical substance released and other release and disposal information. Mark (X) the "Confidential" box next to each item you claim as confidential.

(1) -- Enter the number of each release point identified in the process description, part II, section A, subsection 1d(3).
(2) -- Estimate the amount of the new substance released (a) directly to the environment or (b) into control technology (in kg/day or kg/batch).
(3) -- Mark (X) this column if entries in columns (1) and (2) are confidential business information (CBI).
(4) -- Identify the media (stack air, fugitive air (optional-see Instruction Manual), surface water, on-site or off-site land or incineration, POTW, or other (specify)) to which the new substance will be released from that release point.
(5) -- a. Describe control technology, if any, and control efficiency that will be used to limit the release of the new substance to the environment. For releases disposed of on land, characterize the disposal method and state whether it is approved for disposal of RCRA hazardous waste. On a continuation sheet, for each site describe any additional disposal methods that will be used and whether the waste is subject to secondary or tertiary on-site treatment. b. Estimate the amount released to the environment after control technology (in kg/day).
(6) -- Mark (X) this column if entries in columns (4) and (5) are confidential business information (CBI).
(7) -- Identify the destination(s) of releases to water. Please supply NPDES (National Pollutant Discharge Elimination System) numbers for direct discharges or NPDES numbers of the POTW (Publicly Owned Treatment Works). Mark (X) if the POTW name or NPDES # is confidential business information (CBI).

Release Number	Amount of New Substance Released		CBI	Medium of release e.g. Stack air	Control technology and efficiency (you may wish to optionally attach efficiency data)			CBI
(1)	(2a)	(2b)	(3)	(4)	(5a)	Binding Mark (X)	(5b)	(6)
XXX	XXX	XXX	X	XXX	XXX		XXX	X
XXX	XXX	XXX	X	XXX	XXX		XXX	X
XXX	XXX	XXX	X	XXX	XXX		XXX	X
XXX	XXX	XXX	X	XXX	XXX		XXX	X
XXX	XXX	XXX	X	XXX	XXX		XXX	X

Mark (X) this box if the data continues on the next page. ☐

(7) Mark (X) the destination(s) of releases to water.		NPDES#	CBI
☐ POTW--provide name(s)			☐
☐ Navigable waterway- - provide name(s)			☐
☐ Other--Specify			☐

Enter Attachment filename for Part II, Section A. ☐

Part II-- HUMAN EXPOSURE AND ENVIRONMENTAL RELEASE – Continued

Section B -- INDUSTRIAL SITES CONTROLLED BY OTHERS

The information on pages 10 and 10a refer to consolidated chemical number(s): ☐ **1** ☐ **2** ☐ **3** ☐ **4** ☐ **5** ☐ **6**

Complete section B for typical processing or use operations involving the new chemical substance at sites you do not control. Importers do not have to complete this section for operations outside the U.S.; however, you must report any processing or use activities after import. See the Instructions Manual. *Complete a separate section B for each type of processing, or use operation involving the new chemical substance*. If the same operation is performed at more than one site describe the typical operation common to these sites. Identify additional sites on a continuation sheet.

1(a). Operation Description -- To claim information in this section as confidential, bracket (e.g. {}) the specific information that you claim as confidential.

(1) -- Diagram the major unit operation steps and chemical conversions, including interim storage and transport containers (specify - e.g. 5 gallon pails, 55 gallon drums, rail cars, tank trucks, etc). On the diagram, identify by letter and briefly describe each worker activity.

(2) -- Either in the diagram or in the text field 1(b) below, provide the identity, the approximate weight (by kg/day or kg/batch, on an 100% new chemical substance basis), and entry point of all feedstocks (including reactants, solvents and catalysts, etc) and all products, recycle streams, and wastes. Include cleaning chemicals (note frequency if not used daily or per batch).

(3) -- Either in the diagram or in the text field 1(b) below, identify by number the points of release, including small or intermittent releases, to the environment of the new chemical substance.

(4) -- Please enter the # of sites (remember to identify the locations of these sites on a continuation sheet):

	Number of Sites		Confidential ☐

1(b). (Optional) This space is for a text description to clarify the diagram above. Confidential ☐

Enter Attachment filename for Part II, Section B on the bottom of page 10a.		☐

PMN Page 10a

2. Worker Exposure/Environmental Release

(1) -- From the diagram above, provide the letter for each worker activity. Complete 2-8 for each worker activity described.
(2) -- Estimate the number of workers exposed for all sites combined.
(4) -- Estimate the typical duration of exposure per worker in (a) hours per day and (b) days per year.
(6) -- Describe physical form of exposure and % new chemical substance (if in mixture), and any protective equipment and engineering controls, if any, used to protect workers.
(7) -- Estimate the percent of the new substance as formulated when packaged or used as a final product.
(9) -- From the process diagram above, enter the number of each release point. Complete 9-13 for each release point identified.
(10) -- Estimate the amount of the new substance released (a) directly to the environment or (b) into control technology to the environment (in kg/day or kg/batch).
(12) -- Describe media of release i.e. stack air, fugitive air (optional-see Instructions Manual), surface water, on-site or off-site land or incineration, POTW, or other (specify) and control technology, if any, that will be used to limit the release of the new substance to the environment.
(14) -- Identify byproducts which may result from the operation.
(3), (5), (8), (11), (13) and (15) -- Mark (X) this column if any of the proceeding entries are confidential business information (CBI).

Letter of Activity	# of Workers Exposed	CBI	Duration of Exposure		CBI	Protective Equip./Engineering Controls/Physical Form	% new substance	% in Formulation	CBI
(1)	(2)	(3)	(4a)	(4b)	(5)	(6)	(6)	(7)	(8)

Release Number	Amount of New Substance Released		CBI	Media of Release & Control Technology	CBI
(9)	(10a)	(10b)	(11)	(12)	(13)

Mark (X) this box if the data continues on the next page. ☐

(14) Byproducts: | **(15) CBI** ☐

Enter Attachment filename for Part II, Section B. ☐

OPTIONAL POLLUTION PREVENTION INFORMATION

To claim information in the following section as confidential, bracket (e.g. {}) the specific information that you claim as confidential.

In this section you may provide information not reported elsewhere in this form regarding your efforts to reduce or minimize potential risks associated with activities surrounding manufacturing, processing, use and disposal of the PMN substance. Please include new information pertinent to pollution prevention, including source reduction, recycling activities and safer processes or products available due to the new chemical substance. Source reduction includes the reduction in the amount or toxicity of chemical wastes by technological modification, process and procedure modification, product reformulation, and/or raw materials substitution. Recycling refers to the reclamation of useful chemical components from wastes that would otherwise be treated or released as air emissions or water discharges, or land disposal. Quantitative or qualitative descriptions of pollution prevention, source reduction and recycling should emphasize potential risk reduction in addition to compliance with existing regulatory requirements. The EPA is interested in the information to assess overall net reductions in toxicity or environmental releases and exposures, not the shifting of risks to other media (e.g., air to water) or nonenvironmental areas (e.g., occupational or consumer exposure). To the extent known, information about the technology being replaced will assist EPA in its relative risk determination. In addition, information on the relative cost or performance characteristics of the PMN substance to potential alternatives may be provided.

Describe the expected net benefits, such as

(1) an overall reduction in risk to human health or the environment;
(2) a reduction in the generation of waste materials through recycling, source reduction or other means;
(3) a reduction in the use of hazardous starting materials, reagents, or feedstocks;
(4) a reduction in potential toxicity, human exposure and/or environmental release; or
(5) the extent to which the new chemical substance may be a substitute for an existing substance that poses a greater overall risk to human health or the environment.

Information provided in this section will be taken into consideration during the review of this substance. See PMN Instructions Manual and Pollution Prevention Guidance manual for guidance and examples.

XXX

Enter Attachment filename for Pollution Prevention Page 11.		☐

Part III -- LIST OF ATTACHMENTS

Attach continuation sheets for sections of the form, test data and other data (including physical/chemical properties and structure/activity information), and optional information after this page. Clearly identify the attachment and the section of the form to which it relates, if appropriate. Number consecutively the pages of any paper attachments. In the Number of Pages column below, enter the inclusive page numbers of each attachment for paper submissions or enter the total number of pages for each attachment for electronic submissions. Electronic attachments can be identified by filename.
Mark (X) the "Confidential" box next to any attachment name or filename you claim as confidential. Read the Instructions Manual for guidance on how to claim any information in an attachment as confidential. You must include with the sanitized copy of the notice form a sanitized version of any attachment in which you claim information as confidential.

#	Attachment Name	Attachment Filename	Number of Pages	Associated PMN Section Number	CBI
1	SDS	SDS_3_Redacted.pdf	13	Hazard Information Section (Chemical 869472)	
2	Physical chemical property reports	Physical Chemical Properties_Redacted.pdf	102	Physical and Chemical Properties Worksheet Continued (Chemical	
3	Substance Identity	Substance_ID_Redacted.pdf	42	Physical and Chemical Properties Worksheet Continued (Chemical	
4	Structure Diagram	Structure Diagram_Redacted.pdf	1	Class 1 or 2 Substances Chemical Structure Diagram (Chemical	
5	IES Report	CAS-IES Report_Redacted.pdf	1	Class 1 or 2 Substances ID Method (Chemical 869472)	
6	Manufacturing process diagram.	Process Diagram_Substance 3 _Redacted.pdf	1	Submitter Controlled Operations (Operation 1)	
7	Acute Daphnia	Acute Daphnia_Redacted.pdf	49	Additional Attachments	
8	Acute Oral Toxicity	Acute Study Oral Gavage_Redacted.pdf	36	Additional Attachments	
9	Algae Study	Algae_Redacted.pdf	52	Additional Attachments	
10	AMES	Bacterial Reverse Mutation Assay_Redacted.pdf	62	Additional Attachments	
11	Bovine Corneal Study	BOVINE CORNEAL OPACITY AND	33	Additional Attachments	
12	Predicted Skin Sensitization	DEREK Prediction on Skin	19	Additional Attachments	
13	In Chemico Skin Sensitization	In Chemico Determination of the Skin Sensitization_Redacted.pdf	69	Additional Attachments	
14	Skin Sensitization Early Termination	In vitro Skin Sensitization (Early Termination	3	Additional Attachments	
15	Ready Biodegradability	READY	24	Additional Attachments	
16	Skin Sensitization Weight of Evidence	Weight of Evidence for Skin Sensitization_Redacted.pdf	9	Additional Attachments	
17	Skin Irritation	Skin Irritation_EpiDerm_Redacted.pdf	39	Additional Attachments	
18	Skin corrosion	Skin Corrosion_EpiDerm_Redacted.p	38	Additional Attachments	

Mark (X) this box if the data continues on the next page. ☐

PMN Page 13

PHYSICAL AND CHEMICAL PROPERTIES WORKSHEET

The information on this page refers to chemical number(s): [X] 1 [ ] 2 [ ] 3 [ ] 4 [ ] 5 [ ] 6

To assist EPA's review of physical and chemical properties data, please complete the following worksheet for data you provide and include it in the notice. Identify the property measured, the value of the property, the units in which the property is measured (as necessary), and whether or not the property is claimed as confidential. Give the attachment number (found on page 12) in column (b). The physical state of the neat substance should be provided. These measured properties should be for the neat (100% pure) chemical substance. Properties that are measured for mixtures or formulations should be so noted (% PMN substance in __). You are not required to submit this worksheet; however, EPA strongly recommends that you do so, as it will simplify the review and ensure that confidential information is properly protected. You should submit this worksheet as a supplement to your submission of test data. This worksheet is not a substitute for submission of test data.

Property (a)		Unit	Mark X if Provided	Attachment Number (b)	Value (c)			Measured or Estimate (M or E)	CBI Mark (X) (d)
					(solid)	(liquid)	(gas)		
Physical state of neat substance			[ ]		[ ]	[ ]	[ ]		
Vapor Pressure @ Temperature	XXX	°C	[X]	XXX	XXX		Torr	XXX	X
Density/relative density			[X]	XXX	XXX		g/cm3	XXX	X
Solubility			[ ]				g/L		
@ Temperature		°C							
Solvent									
Solubility in Water @ Temperature	XXX	°C	[X]	XXX	XXX		g/L	XXX	X
Melting Temperature			[X]	XXX	XXX		°C	XXX	X
Boiling / Sublimation temperature @	XXX	Torr	[X]	XXX	XXX		°C	XXX	X
Spectra			[ ]						
Dissociation constant			[ ]						
Octanol / water partition coefficient			[X]	XXX	XXX			XXX	X
Henry's Law constant			[ ]						
Volatilization from water			[ ]						
Volatilization from soil			[ ]						
pH@ concentration			[ ]						
Flammability			[ ]						
Explodability			[ ]						
Adsorption / Coefficient			[ ]						
Particle Size Distribution			[ ]						
Other – Specify	XXX		[X]	XXX	XXX			XXX	X

Continuation Sheet

ID		Field	

PHYSICAL AND CHEMICAL PROPERTIES WORKSHEET

Property (a)		Mark X if Provided	Attachment Number (b)	Value (c)	Measured or Estimate (M or E)	CBI Mark (X) (d)
Other – Specify	XXX	X	XXX	XXX	XXX	X
Other – Specify	XXX	X	XXX	XXX	XXX	X
Other – Specify		☐				
Other – Specify		☐				
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CAS
A DIVISION OF THE
AMERICAN CHEMICAL SOCIETY

InventoryExpertService

Phone: 800-631-1884, 614-447-3870
Fax: 614-447-3747
E-mail: answers@cas.org
Web: cas.org/services/knowledge

INVENTORY EXPERT SERVICE REPORT

Please print the above CA Index Name on the appropriate page of your PMN.

☐ If this box is checked, CAS has made correction(s) marked in red to your IES order. Please make the same corrections to your PMN before submitting it to the EPA.

Recommended use of the chemical and restrictions on use

Recommended use	:	Chemical intermediate
Restrictions on use	:	This product is for experimental uses only. The product has not been completely analyzed and all of the hazards may not be known. Please use caution while handling this product.

SECTION 2. HAZARDS IDENTIFICATION

GHS classification in accordance with 29 CFR 1910.1200

Flammable liquids	:	Category 2

GHS label elements

Hazard pictograms :

Signal Word	:	Danger
Hazard Statements	:	H225 Highly flammable liquid and vapor.
Precautionary Statements	:	**Prevention:** P210 Keep away from heat/sparks/open flames/hot surfaces. No smoking. P233 Keep container tightly closed. P241 Use explosion-proof electrical/ ventilating/ lighting/ equipment. P242 Use only non-sparking tools.

P243 Take precautionary measures against static discharge.
P280 Wear protective gloves/ eye protection/ face protection.

Response:

P303 + P361 + P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower.

Storage:

P403 + P235 Store in a well-ventilated place. Keep cool.

Disposal:

P501 Dispose of contents/ container to an approved waste disposal plant.

Other hazards

Vapors may form explosive mixture with air.

SECTION 3. COMPOSITION/INFORMATION ON INGREDIENTS

Substance / Mixture	:	Substance

Components

No hazardous ingredients

SECTION 4. FIRST AID MEASURES

If inhaled	:	If inhaled, remove to fresh air. Get medical attention if symptoms occur.
In case of skin contact	:	In case of contact, immediately flush skin with plenty of water. Remove contaminated clothing and shoes.
In case of eye contact	:	Flush eyes with water as a precaution. Get medical attention if irritation develops and persists.
If swallowed	:	If swallowed, DO NOT induce vomiting. Get medical attention if symptoms occur. Rinse mouth thoroughly with water.
Most important symptoms and effects, both acute and delayed	:	None known.
Protection of first-aiders	:	No special precautions are necessary for first aid responders.
Notes to physician	:	Treat symptomatically and supportively.

SECTION 5. FIRE-FIGHTING MEASURES

Suitable extinguishing media	:	Water spray Alcohol-resistant foam Carbon dioxide (CO2) Dry chemical
Unsuitable extinguishing media	:	High volume water jet
Specific hazards during fire fighting	:	Do not use a solid water stream as it may scatter and spread fire. Flash back possible over considerable distance. Vapors may form explosive mixtures with air. Exposure to combustion products may be a hazard to health.
Hazardous combustion products	:	Hydrogen fluoride carbonyl fluoride potentially toxic fluorinated compounds aerosolized particulates Carbon oxides
Specific extinguishing methods	:	Use extinguishing measures that are appropriate to local circumstances and the surrounding environment. Use water spray to cool unopened containers. Remove undamaged containers from fire area if it is safe to do so. Evacuate area.
Special protective equipment for fire-fighters	:	Wear self-contained breathing apparatus for firefighting if necessary. Use personal protective equipment.

SECTION 6. ACCIDENTAL RELEASE MEASURES

Personal precautions, protective equipment and emergency procedures	:	Remove all sources of ignition. Ventilate the area. Follow safe handling advice and personal protective equipment recommendations.
Environmental precautions	:	Discharge into the environment must be avoided. Prevent further leakage or spillage if safe to do so. Prevent spreading over a wide area (e.g., by containment or oil barriers). Retain and dispose of contaminated wash water. Local authorities should be advised if significant spillages cannot be contained.
Methods and materials for containment and cleaning up	:	Non-sparking tools should be used. Soak up with inert absorbent material. Suppress (knock down) gases/vapors/mists with a water spray jet. For large spills, provide diking or other appropriate contain-

ment to keep material from spreading. If diked material can be pumped, store recovered material in appropriate container.
Clean up remaining materials from spill with suitable absorbent.
Local or national regulations may apply to releases and disposal of this material, as well as those materials and items employed in the cleanup of releases. You will need to determine which regulations are applicable.
Sections 13 and 15 of this SDS provide information regarding certain local or national requirements.

SECTION 7. HANDLING AND STORAGE

Technical measures	:	See Engineering measures under EXPOSURE CONTROLS/PERSONAL PROTECTION section.
Local/Total ventilation	:	If sufficient ventilation is unavailable, use with local exhaust ventilation. If advised by assessment of the local exposure potential, use only in an area equipped with explosion-proof exhaust ventilation.
Advice on safe handling	:	Handle in accordance with good industrial hygiene and safety practice, based on the results of the workplace exposure assessment Non-sparking tools should be used. Keep container tightly closed. Keep away from heat and sources of ignition. Take precautionary measures against static discharges. Take care to prevent spills, waste and minimize release to the environment.
Conditions for safe storage	:	Keep in properly labeled containers. Keep tightly closed. Keep in a cool, well-ventilated place. Store in accordance with the particular national regulations. Keep away from heat and sources of ignition.
Materials to avoid	:	Do not store with the following product types: Strong oxidizing agents Organic peroxides Flammable solids Pyrophoric liquids Pyrophoric solids Self-heating substances and mixtures Substances and mixtures which in contact with water emit flammable gases Explosives Gases

SECTION 8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Ingredients with workplace control parameters

Contains no substances with occupational exposure limit values.

Occupational exposure limits of decomposition products

Components	CAS-No.	Value type (Form of exposure)	Control parameters / Permissible concentration	Basis
Hydrofluoric acid	7664-39-3	TWA	3 ppm 2.5 mg/m³	NIOSH REL
		C	6 ppm 5 mg/m³	NIOSH REL
		TWA	3 ppm	OSHA Z-2
		TWA	0.5 ppm (Fluorine)	ACGIH
		C	2 ppm (Fluorine)	ACGIH
Carbonyl difluoride	353-50-4	TWA	2 ppm	ACGIH
		STEL	5 ppm	ACGIH
		ST	5 ppm 15 mg/m³	NIOSH REL
		TWA	2 ppm 5 mg/m³	NIOSH REL
Carbon dioxide	124-38-9	TWA	5,000 ppm	ACGIH
		STEL	30,000 ppm	ACGIH
		TWA	5,000 ppm 9,000 mg/m³	OSHA Z-1
		TWA	5,000 ppm 9,000 mg/m³	NIOSH REL
		ST	30,000 ppm 54,000 mg/m³	NIOSH REL
Carbon monoxide	630-08-0	TWA	25 ppm	ACGIH
		TWA	35 ppm 40 mg/m³	NIOSH REL
		C	200 ppm 229 mg/m³	NIOSH REL
		TWA	50 ppm 55 mg/m³	OSHA Z-1

Engineering measures : Processing may form hazardous compounds (see section 10).
Minimize workplace exposure concentrations.
If sufficient ventilation is unavailable, use with local exhaust ventilation.
If advised by assessment of the local exposure potential, use only in an area equipped with explosion-proof exhaust ventilation.

Personal protective equipment

Respiratory protection	:	General and local exhaust ventilation is recommended to maintain vapor exposures below recommended limits. Where concentrations are above recommended limits or are unknown, appropriate respiratory protection should be worn. Follow OSHA respirator regulations (29 CFR 1910.134) and use NIOSH/MSHA approved respirators. Protection provided by air purifying respirators against exposure to any hazardous chemical is limited. Use a positive pressure air supplied respirator if there is any potential for uncontrolled release, exposure levels are unknown, or any other circumstance where air purifying respirators may not provide adequate protection.
Hand protection		
Material	:	Neoprene
Remarks	:	Choose gloves to protect hands against chemicals depending on the concentration specific to place of work. For special applications, we recommend clarifying the resistance to chemicals of the aforementioned protective gloves with the glove manufacturer. Wash hands before breaks and at the end of workday. Breakthrough time is not determined for the product. Change gloves often!
Eye protection	:	Wear the following personal protective equipment: Safety glasses
Skin and body protection	:	Select appropriate protective clothing based on chemical resistance data and an assessment of the local exposure potential. Wear the following personal protective equipment: If assessment demonstrates that there is a risk of explosive atmospheres or flash fires, use flame retardant antistatic protective clothing. Skin contact must be avoided by using impervious protective clothing (gloves, aprons, boots, etc).
Hygiene measures	:	If exposure to chemical is likely during typical use, provide eye flushing systems and safety showers close to the working place. When using do not eat, drink or smoke. Wash contaminated clothing before re-use.

SECTION 9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance	:	liquid

Color	:	colorless
Odor	:	odorless
Odor Threshold	:	No data available
pH	:	No data available
Evaporation rate	:	No data available
Flammability (solid, gas)	:	Not applicable
Flammability (liquids)	:	Ignitable (see flash point)
Upper explosion limit / Upper flammability limit	:	No data available
Lower explosion limit / Lower flammability limit	:	No data available
Relative vapor density	:	No data available
Decomposition temperature	:	No data available
Viscosity		
Viscosity, kinematic	:	No data available
Explosive properties	:	Not explosive

Oxidizing properties	:	The substance or mixture is not classified as oxidizing.
Particle size	:	Not applicable

SECTION 10. STABILITY AND REACTIVITY

Reactivity	:	Not classified as a reactivity hazard.
Chemical stability	:	Stable under normal conditions.
Possibility of hazardous reactions	:	Highly flammable liquid and vapor. Vapors may form explosive mixture with air. Can react with strong oxidizing agents. Hazardous decomposition products will be formed at elevated temperatures.
Conditions to avoid	:	Heat, flames and sparks.
Incompatible materials	:	Oxidizing agents

Hazardous decomposition products

Thermal decomposition	:	Hydrofluoric acid Carbonyl difluoride Carbon dioxide Carbon monoxide

SECTION 11. TOXICOLOGICAL INFORMATION

Information on likely routes of exposure

Inhalation
Skin contact
Ingestion
Eye contact

Acute toxicity

Not classified based on available information.

Skin corrosion/irritation

Not classified based on available information.

Serious eye damage/eye irritation

Not classified based on available information.

Respiratory or skin sensitization

Skin sensitization

Not classified based on available information.

Respiratory sensitization

Not classified based on available information.

Germ cell mutagenicity

Not classified based on available information.

Carcinogenicity

Not classified based on available information.

IARC No ingredient of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

OSHA No component of this product present at levels greater than or equal to 0.1% is on OSHA's list of regulated carcinogens.

NTP No ingredient of this product present at levels greater than or equal to 0.1% is identified as a known or anticipated carcinogen by NTP.

Reproductive toxicity

Not classified based on available information.

STOT-single exposure

Not classified based on available information.

STOT-repeated exposure

Not classified based on available information.

Aspiration toxicity

Not classified based on available information.

SECTION 12. ECOLOGICAL INFORMATION

Ecotoxicity

No data available

Persistence and degradability

No data available

Bioaccumulative potential

No data available

Mobility in soil

No data available

Other adverse effects

No data available

SECTION 13. DISPOSAL CONSIDERATIONS

Disposal methods

Waste from residues	:	Dispose of in accordance with local regulations.
Contaminated packaging	:	Empty containers should be taken to an approved waste handling site for recycling or disposal. Empty containers retain residue and can be dangerous. Do not pressurize, cut, weld, braze, solder, drill, grind, or expose such containers to heat, flame, sparks, or other sources

of ignition. They may explode and cause injury and/or death.
If not otherwise specified: Dispose of as unused product.

SECTION 14. TRANSPORT INFORMATION

International Regulations

UNRTDG

UN number	:	UN 1993
Proper shipping name	:	FLAMMABLE LIQUID, N.O.S.
Class	:	3
Packing group	:	II
Labels	:	3

IATA-DGR

UN/ID No.	:	UN 1993
Proper shipping name	:	Flammable liquid, n.o.s.
Class	:	3
Packing group	:	II
Labels	:	Flammable Liquids
Packing instruction (cargo aircraft)	:	364
Packing instruction (passenger aircraft)	:	353

IMDG-Code

UN number	:	UN 1993
Proper shipping name	:	FLAMMABLE LIQUID, N.O.S.
Class	:	3
Packing group	:	II
Labels	:	3
EmS Code	:	F-E, S-E
Marine pollutant	:	yes

Transport in bulk according to Annex II of MARPOL 73/78 and the IBC Code

Not applicable for product as supplied.

Domestic regulation

49 CFR

UN/ID/NA number	:	UN 1993
Proper shipping name	:	Flammable liquids, n.o.s.
Class	:	3
Packing group	:	II
Labels	:	FLAMMABLE LIQUID
ERG Code	:	128

Marine pollutant : yes(

Special precautions for user

The transport classification(s) provided herein are for informational purposes only, and solely based upon the properties of the unpackaged material as it is described within this Safety Data Sheet. Transportation classifications may vary by mode of transportation, package sizes, and variations in regional or country regulations.

SECTION 15. REGULATORY INFORMATION

EPCRA - Emergency Planning and Community Right-to-Know

CERCLA Reportable Quantity

This material does not contain any components with a CERCLA RQ.

SARA 304 Extremely Hazardous Substances Reportable Quantity

This material does not contain any components with a section 304 EHS RQ.

SARA 302 Extremely Hazardous Substances Threshold Planning Quantity

This material does not contain any components with a section 302 EHS TPQ.

SARA 311/312 Hazards : Flammable (gases, aerosols, liquids, or solids)

SARA 313 : This material does not contain any chemical components with known CAS numbers that exceed the threshold (De Minimis) reporting levels established by SARA Title III, Section 313.

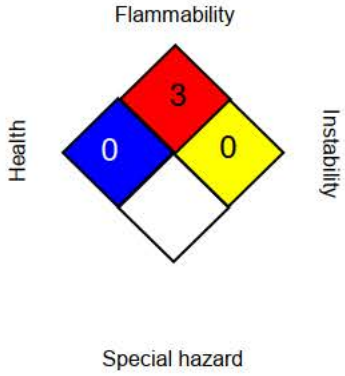
US State Regulations

Pennsylvania Right To Know

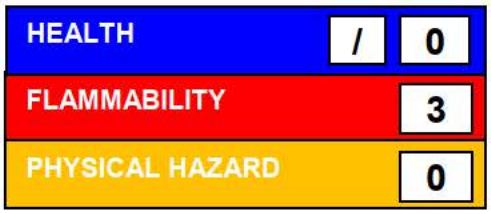
SECTION 16. OTHER INFORMATION

Further information

NFPA 704:



HMIS® IV:



HMIS® ratings are based on a 0-4 rating scale, with 0 representing minimal hazards or risks, and 4 representing significant hazards or risks. The "*" represents a chronic hazard, while the "/" represents the absence of a chronic hazard.

Full text of other abbreviations

ACGIH	:	USA. ACGIH Threshold Limit Values (TLV)
NIOSH REL	:	USA. NIOSH Recommended Exposure Limits
OSHA Z-1	:	USA. Occupational Exposure Limits (OSHA) - Table Z-1 Limits for Air Contaminants
OSHA Z-2	:	USA. Occupational Exposure Limits (OSHA) - Table Z-2
ACGIH / TWA	:	8-hour, time-weighted average
ACGIH / STEL	:	Short-term exposure limit
ACGIH / C	:	Ceiling limit
NIOSH REL / TWA	:	Time-weighted average concentration for up to a 10-hour workday during a 40-hour workweek
NIOSH REL / ST	:	STEL - 15-minute TWA exposure that should not be exceeded at any time during a workday
NIOSH REL / C	:	Ceiling value not be exceeded at any time.
OSHA Z-1 / TWA	:	8-hour time weighted average
OSHA Z-2 / TWA	:	8-hour time weighted average

AICS - Australian Inventory of Chemical Substances; ASTM - American Society for the Testing of Materials; bw - Body weight; CERCLA - Comprehensive Environmental Response, Compensation, and Liability Act; CMR - Carcinogen, Mutagen or Reproductive Toxicant; DIN - Standard of the German Institute for Standardisation; DOT - Department of Transportation; DSL - Domestic Substances List (Canada); ECx - Concentration associated with x% response; EHS - Extremely Hazardous Substance; ELx - Loading rate associated with x% response; EmS - Emergency Schedule; ENCS - Existing and New Chemical Substances (Japan); ErCx - Concentration associated with x% growth rate response; ERG - Emergency Response Guide; GHS - Globally Harmonized System; GLP - Good Laboratory Practice; HMIS - Hazardous Materials Identification System; IARC - International Agency for Research on Cancer; IATA - International Air Transport Association; IBC

- International Code for the Construction and Equipment of Ships carrying Dangerous Chemicals in Bulk; IC50 - Half maximal inhibitory concentration; ICAO - International Civil Aviation Organization; IECSC - Inventory of Existing Chemical Substances in China; IMDG - International Maritime Dangerous Goods; IMO - International Maritime Organization; ISHL - Industrial Safety and Health Law (Japan); ISO - International Organisation for Standardization; KECI - Korea Existing Chemicals Inventory; LC50 - Lethal Concentration to 50 % of a test population; LD50 - Lethal Dose to 50% of a test population (Median Lethal Dose); MARPOL - International Convention for the Prevention of Pollution from Ships; MSHA - Mine Safety and Health Administration; n.o.s. - Not Otherwise Specified; NFPA - National Fire Protection Association; NO(A)EC - No Observed (Adverse) Effect Concentration; NO(A)EL - No Observed (Adverse) Effect Level; NOELR - No Observable Effect Loading Rate; NTP - National Toxicology Program; NZIoC - New Zealand Inventory of Chemicals; OECD - Organization for Economic Co-operation and Development; OPPTS - Office of Chemical Safety and Pollution Prevention; PBT - Persistent, Bioaccumulative and Toxic substance; PICCS - Philippines Inventory of Chemicals and Chemical Substances; (Q)SAR - (Quantitative) Structure Activity Relationship; RCRA - Resource Conservation and Recovery Act; REACH - Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals; RQ - Reportable Quantity; SADT - Self-Accelerating Decomposition Temperature; SARA - Superfund Amendments and Reauthorization Act; SDS - Safety Data Sheet; TCSI - Taiwan Chemical Substance Inventory; TSCA - Toxic Substances Control Act (United States); UN - United Nations; UNRTDG - United Nations Recommendations on the Transport of Dangerous Goods; vPvB - Very Persistent and Very Bioaccumulative

Sources of key data used to compile the Material Safety Data Sheet	:	Internal technical data, data from raw material SDSs, OECD eChem Portal search results and European Chemicals Agency, http://echa.europa.eu/
Revision Date	:	11/14/2019

The information provided in this Safety Data Sheet is correct to the best of our knowledge, information and belief at the date of its publication. The information is designed only as a guidance for safe handling, use, processing, storage, transportation, disposal and release and shall not be considered a warranty or quality specification of any type. The information provided relates only to the specific material identified at the top of this SDS and may not be valid when the SDS material is used in combination with any other materials or in any process, unless specified in the text. Material users should review the information and recommendations in the specific context of their intended manner of handling, use, processing and storage, including an assessment of the appropriateness of the SDS material in the user's end product, if applicable.

US / Z8

Entire Submission Confidential Business Information

TRADE SECRET

Study Title

CAS # A 48-HOUR STATIC ACUTE TOXICITY TEST WITH THE CLADOCERAN (*DAPHNIA MAGNA*)

Test Guidelines

OECD Guideline 202 (2004)
U.S. EPA OCSPP 850.1010 (2016)

Authors

Amanda K Gerke, B.S. (Study Director)
Laura A. Lockard, M.S.
Suzanne Z. Schneider, Ph.D.
Ling Zhang, Ph.D.

Study Completion Date

October 17, 2018

Test Facility

EAG, Inc.
8598 Commerce Drive
Easton, Maryland 21601
U.S.A.

Performing Laboratory Study Number

783A-103A

Good Laboratory Practice Compliance Statement

The study described in this final report was conducted in compliance with U.S. EPA Good Laboratory Practice Standards (40 CFR Parts 160 and 792) that are compatible with:

- OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17)
- Japan MAFF (11 Nousan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999) with the following exception:

  Periodic analyses of the water and feed for potential contaminants were not performed according to Good Laboratory Practice Standards but were performed using a certified laboratory and standard U.S. EPA analytical methods.

Study Director

Amanda K. Gerke — 17 October 2018

Amanda K Gerke, B.S.
Staff Scientist II
EAG Laboratories-Easton

Date

Quality Assurance Statement

Study Number

783A-103A

Study Title

A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

Activity	*Audit Dates**	*Dates Findings Reported to Study Director*	*Dates Findings Reported to Management*
Protocol	February 14, 2018	February 14, 2018	February 15, 2018
Initial Trial (783A-103) Test Substance Preparation	June 7, 2018	June 7, 2018	June 7, 2018
Definitive Test (783A-103A) Test Substance Preparation	August 21, 2018	August 21, 2018	September 6, 2018
pH Measurements	August 23, 2018	August 23, 2018	August 24, 2018
Analytical Data and Draft Report	September 25-27, 2018	October 8, 2018	September 28, 2018
Biological Data and Draft Report	September 6, 2018	September 7, 2018	October 9, 2018
Final Report	October 17, 2018	October 17, 2018	October 17, 2018

* All inspections were study-based unless otherwise noted.

These inspections confirm that the methods, procedures, and observations are accurately and completely described in this final report, and the reported results accurately and completely reflect the raw data of this study.

Jessica J. Anders — 17 Oct 2018

Jessica T. Anders
Senior Quality Assurance Representative
EAG Laboratories-Easton

Date

Certification of Authenticity

A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

We, the undersigned, declare that the work described in this final report was performed under our supervision, and that this final report provides an accurate record of the procedures and results.

Report by:

Amanda K. Gerke, B.S. (Study Director) — 17 October 2018
Staff Scientist II — Date
EAG Laboratories-Easton

for Laura A. Lockard — Oct 17, 2018
Laura A. Lockard, M.S. (Principal Analyst) — Date
Staff Scientist II
EAG Laboratories-Easton

Approved by:

Suzanne Z. Schneider — 17 October 2018
Suzanne Z. Schneider, Ph.D. — Date
Manager of Aquatic Toxicology
EAG Laboratories-Easton

Oct 17, 2018
Ling Zhang, Ph.D. — Date
Manager of Analytical Chemistry
EAG Laboratories-Easton

Study Initiated:
June 1, 2018

Date Study Completed:
October 17, 2018

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Basic Study Information

Study Title

CAS # A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

Study Objective

The objective of this study was to determine the acute effects of CAS # on the cladoceran, *Daphnia magna*, during a 48-hour exposure period under closed-system, static test conditions.

Study Director

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Laboratory Management, EAG Laboratories-Easton

Suzanne Z. Schneider, Ph.D., Manager of Aquatic Toxicology
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Test Item

CAS #

Testing Facilities

EAG, Inc.
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U.S.A.

CAS # A 48-HOUR STATIC ACUTE TOXICITY TEST WITH THE CLADOCERAN (*DAPHNIA MAGNA*)

1.0 Summary

STUDY: CAS # A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

EAG LABORATORIES PROJECT NUMBER: 783A-103A

TEST DATES: Experimental Start (OECD) – June 7, 2018
Experimental Start (EPA) – August 21, 2018
Biological Termination – August 23, 2018
Experimental Termination – August 27, 2018

LENGTH OF EXPOSURE: 48 Hours

TEST ORGANISMS: Cladoceran (*Daphnia magna*)

SOURCE OF TEST ORGANISMS: EAG Laboratories-Easton cultures

TEST CONCENTRATIONS:

Nominal Concentration
Negative Control
1.3 mg a.i./L
2.5 mg a.i./L
5.0 mg a.i./L
10 mg a.i./L
20 mg a.i./L

RESULTS:	Nominal Concentration
48-Hour EC_{50}:	3.7 mg a.i./L

The highest nominal concentration causing no immobility at test end was 2.5 mg a.i./L. The lowest nominal concentration causing 100% immobility at test end was 10 mg a.i./L. The NOEC was 2.5 mg a.i./L.

2.0 Introduction

This study was conducted by EAG Laboratories for at the EAG Laboratories aquatic toxicology facility in Easton, Maryland. The initial test trial was conducted from June 7 to 9, 2018 but was repeated due to immobility data that would not yield an EC_{50} calculation. The in-life phase of the definitive test was conducted from August 21 to 23, 2018. Raw data generated by EAG Laboratories-Easton and a copy of the final report are filed under Project Number 783A-103A in archives located on the EAG Laboratories site in Easton, Maryland.

3.0 Objective

The objective of this study was to determine the acute effects of CAS # on the cladoceran (*Daphnia magna*), during a 48-hour exposure period under closed-system, static test conditions.

4.0 Experimental Design

Neonates < 24 hours old at test start were exposed to a geometric series of five test concentrations and negative control (dilution water) for 48 hours under closed-system, static conditions. Four replicate test chambers were maintained in each treatment and control group, with five daphnids in each test chamber, for a total of 20 daphnids per concentration. Nominal test concentrations were selected in consultation with the Sponsor based on exploratory range-finding toxicity data and the reported water solubility. Nominal test concentrations selected for the definitive test were 1.3, 2.5, 5.0, 10 and 20 mg a.i./L. Test concentrations were measured in samples of test water collected from each treatment and control group at the beginning and at the end of the test.

Daphnia were indiscriminately assigned to test chambers at test initiation. Observations of immobility and other signs of toxicity were made approximately 3.5, 24 and 48 hours after test initiation. Cumulative percent immobility observed in the treatment groups was used to determine EC_{50} values at 24 and 48 hours. The no-observed-effect concentration (NOEC) was determined by statistical anlysis of the immobility data. The highest nominal test concentration causing no immobility at test end and the lowest nominal test concentration causing 100% immobility at test end were assessed by visually interpreting the immobility and clinical observation data.

5.0 Materials and Methods

The study was conducted according to the procedures outlined in the protocol, "CAS # A 48-hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*)". The protocol was based on procedures outlined in the OECD Guidelines for Testing of Chemicals, Guideline 202: *Daphnia sp., Acute Immobilization Test* (1); U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines, OCSPP 850.1010: *Aquatic Invertebrate Acute Toxicity Test, Freshwater*

Daphnids (2); and ASTM Standard E 729-96: *Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians* (3).

5.1 Test Substance

The test substance used to prepare the test solutions, the analytical matrix fortification samples and the analytical calibration standards for the study was received from on April 6, 2018. It was assigned EAG Laboratories identification number 14633 upon receipt and was stored under ambient conditions. The test substance, a liquid, was identified as: Lot number Sample# 12639; CAS # The test substance contained 99.09% active ingredient (Appendix 1).

5.2 Test Organism

The cladoceran (*Daphnia magna*) was selected as the test species for this study. Daphnids are representative of an important group of aquatic invertebrates and were selected for use in the test based upon past history of use in the laboratory. Daphnid neonates used in the test were less than 24 hours old and were obtained from cultures maintained by EAG Laboratories, Easton, Maryland.

Adult daphnids were cultured in water from the same source and at approximately the same temperature as used during the test. During the 2-week period immediately preceding the test, water temperatures in the cultures ranged from 19.8 to 20.4ºC, measured with a digital thermometer. The pH of the water ranged from 8.1 to 8.6, measured with a Thermo Orion Benchtop 4 Star Plus pH/ISE meter. Dissolved oxygen remained ≥6.9 mg/L (≥76% of saturation), measured with a Thermo Orion Benchtop 3 Star Plus dissolved oxygen meter. Daphnids in the cultures were fed daily a mixture of yeast, cereal grass media and trout chow (YCT), as well as a suspension of the freshwater green alga, *Raphidocelis subcapitata*, formally (*Pseudokirchneriella subcapitata*), and a supplemental vitamin stock. The adults were fed prior to test initiation, but neonates were not fed during the test. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in YCT are presented in Appendix 2.

The five adult daphnids used to supply neonates for the test were held 27 days prior to collection of the neonates for testing and had each produced at least six previous broods. Adult daphnids in the culture had produced an average of at least three young per adult per day over the 7-day period prior to the test. The adults showed no signs of disease or stress, no ephippia were produced during the holding period, and mortality in the culture stock was less than 10% in the two-day period prior to test initiation. At test initiation, the neonate daphnids were collected from the cultures and indiscriminately transferred one or two at a time to transfer containers until each container contained its compliment of five daphnids. Each group of daphnids then was transferred to an indiscriminately assigned test compartment. All transfers were made below the water surface using a wide-bore pipette.

Loading in each test chamber during the test was approximately 52 mL of test solution per daphnid.

5.3 Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the EAG Laboratories-Easton site. The well water was passed through a sand filter to remove particles greater than approximately 25 µm and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the water was filtered to 0.45 µm and passed through an ultraviolet sterilizer to remove fine particles and microorganisms.

The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity, pH and total organic carbon (TOC) of the well water during the approximate four-week period immediately preceding the test are presented in Appendix 3. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the well water are presented in Appendix 4.

5.4 Test Apparatus

Test chambers were 250 mL glass French Square bottles containing approximately 260 mL of test solution. The depth of the test water in a representative chamber was 13.0 cm. All test chambers were labeled with the project number, test concentration, and replicate designation. Test chambers were impartially positioned in an environmental chamber set to maintain the desired test temperature throughout the exposure period.

5.5 Non-GLP Range-Finding Test

The range-finding study was conducted at nominal concentrations of 0.95, 3.8, 15 and 61 mg a.i./L for 48 hours under static test conditions. Percent immobility in the 0.95, 3.8, 15 and 61 mg a.i./L treatment groups at test termination was 0, 0, 100 and 100%, respectively. No sublethal effects were observed at test termination (Table 1).

5.6 Preparation of Test Concentrations

Test solutions were prepared by direct addition of calculated volumes of test substance with Hamilton gas-tight syringes in 2 or 4 L of dilution water at nominal concentrations of 1.3, 2.5, 5.0, 10 and 20 mg a.i./L. The calculated amounts of test substance were determined based on the reported density of the test substance (1.6194 g/cm^3). The stock solution concentration was adjusted to 100% active ingredient during preparation, based on the reported test substance purity (99.09%). The test substance was dispensed into a glass beaker, blanketed with nitrogen and covered with parafilm to help reduce volatility. The solutions were nearly brought to final volume and the test substance was added. The solutions were then brought to final volume, stirred with a Teflon® lined stir bar on a magnetic stir plate for approximately 15 minutes and sonicated for approximately five minutes. The test

solutions appeared clear and colorless with no precipitates. The negative control solution was dilution water only.

5.7 Analytical Sampling

Test water samples were collected from the batches of test solution prepared for each treatment and control group at the beginning of the test, and from two of the four replicate test chambers in each treatment and control group in each treatment and control group at 48 hours (± 1 hour) to measure concentrations of the test substance. Samples (10.0 mL) were collected from mid-depth, placed in glass vials with Teflon-lined caps containing 10.0 mL of toluene and processed immediately for analysis.

5.8 Analytical Method

Freshwater samples of CAS # were analyzed by gas chromatography with electron capture detection (GC/ECD). A method outline is provided in Appendix 5, Figure 1.

Typical GC/MS Instrument and Conditions

INSTRUMENT:	Agilent model 7890A gas chromatograph (GC)
DETECTOR:	Electron Capture Detector (ECD)
ANALYTICAL COLUMN:	Agilent GS-Q analytical column (30 m x 0.32 mm x 1.4 µm)
INJECTOR TEMPERATURE:	220°C
RUN TIME:	11.2 minutes
OVEN:	Initial temperature: 45°C Initial time: 1.00 minute Ramp: 25°C/minute Final temperature: 250°C Final hold time: 2.00 minute
DETECTOR TEMPERATURE:	250°C
INJECTION VOLUME:	2 µL
CARRIER GAS:	Helium, 12 psi
MAKEUP GAS:	Nitrogen
APPROXIMATE RETENTION TIME:	7.75 minutes

Calibration standards of CAS # ranging in concentration from 0.200 to 25.0 mg a.i./L, were prepared in toluene using a stock solution of CAS # in acetone. A calibration curve was constructed for each set of analysis. The peak area and the theoretical concentrations of the calibration standards were fit with least-squares regression analysis to a weighted (1/x) linear function. The concentrations of CAS # in the samples were determined by substituting the peak area responses of the samples into the applicable linear regression equations. Samples were diluted, as necessary, into the calibration standard range using dilution factors ranging from 1.00 to 3.00.

The method limit of quantitation (LOQ) for these analyses was set at 0.400 mg a.i./L, based upon the lowest analyte concentration in a fortified sample. Matrix blank samples were analyzed to determine possible interferences.

The signal-to-noise (S/N) ratios for four injections of the lowest calibration standard were determined. The limit of detection (LOD) was calculated for each standard by dividing the standard concentration by the S/N ratio x 3 x the dilution factor (1.00) of the matrix blank samples. The mean LOD for CAS # was calculated and reported as 0.00502 mg a.i./L.

Samples were fortified at 0.400, 1.00, and 50.0 mg a.i./L using stock solutions of CAS # in acetone and were analyzed for CAS # with the sample sets.

Example Calculations

An example of a typical calculation used in the analysis of CAS # for sample number 783A-103A-6 with a nominal concentration of 20 mg a.i./L follows:

Concentration of CAS # in sample (mg a.i./L) =

$$\frac{\text{peak area - (y-intercept)}}{\text{slope}} \text{ x dilution factor}$$

$$\text{Percent of nominal concentration} = \frac{\text{Measured concentration of sample (mg a.i./L)}}{\text{Nominal concentration of sample (mg a.i./L)}} \text{ x } 100$$

Peak Area = 34534.8

Y-Intercept = 365.435

Slope = 10369

Dilution Factor = 1.00

Concentration of CAS # in sample (mg a.i./L) = $\frac{34534.8 - 365.435}{10369}$ x 1.00

Concentration of CAS # in sample (mg a.i./L) = 3.30*

CAS # concentration = $\frac{3.30 \text{ mg a.i./L}}{20 \text{ mg a.i./L}}$ x 100

Percent of nominal CAS # concentration = 16.5%*

* Results were generated using Excel 2010 in full precision mode. Manual calculations may differ slightly.

Analytical Stocks and Standards Preparation

A stock solution of CAS # was prepared by accurately measuring 625 µL of the test substance (based on a reported test substance density of 1.6194 g/mL and corrected for 99.09% purity of the test substance) using a gas-tight syringe. The test substance was transferred to a 100 mL volumetric flask and the contents were brought to volume using acetone. The primary stock solution (10.0 mg a.i./mL) was diluted in acetone to prepare a 1.00 mg a.i./mL stock solution. The 1.00 mg a.i./mL stock solution was diluted in acetone to prepare a 0.200 mg a.i./mL stock solution. The 0.200 mg a.i./mL stock solution was diluted in acetone to prepare a 0.100 mg a.i./mL stock solution. The 0.200 and 10.0 mg a.i./mL stock solutions were used to fortify the quality control samples. The 1.00 and 0.100 mg a.i./mL stock solutions were used to prepare the calibration standards in toluene. The following shows the dilution scheme for a set of calibration standards:

Stock Concentration (mg a.i./mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg a.i./L)
0.100	20.0	10.0	0.200
0.100	100	10.0	1.00
1.00	50.0	10.0	5.00
1.00	100	10.0	10.0
1.00	250	10.0	25.0

5.9 Environmental Conditions

Fluorescent light bulbs that emit wavelengths similar to natural sunlight were used for illumination of the test chambers. A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity at test initiation was 601 lux at the surface of the water of one representative test chamber and was measured using a SPER Scientific Model 840006 light meter.

The target test temperature during the study was 20 ± 1ºC. Temperature was measured in each test chamber at the beginning of the test and at the end of the test using a digital thermometer. Temperature was also measured continuously in a beaker of water adjacent to the test chambers using an AmegaView centralized

monitoring system, which was verified prior to test initiation with a digital thermometer.

Dissolved oxygen and pH were measured in each test chamber at the beginning of the test and at the end of the test. Dissolved oxygen was measured using a Thermo Scientific Orion Star A213 Benchtop RDO/DO meter. Measurements of pH were made using a Thermo Scientific Orion DUAL STAR pH/ISE meter.

Hardness, alkalinity and specific conductance were measured at the beginning of the test in the dilution water. Hardness and alkalinity measurements were made by titration based on procedures in Standard Methods for the Examination of Water and Wastewater (4). Specific conductance was measured using a Thermo Scientific Orion Star A122 conductivity meter.

5.10 Observations

Observations of immobilization and clinical signs of toxicity were made at approximately 3.5, 24 and 48 hours (± 1 hour). Daphnids that were not able to swim within 15 seconds after gentle agitation of the test vessel were considered immobilized (even if they could still move their antennae). All observations of abnormal behavior were noted. Due to the volatile nature of the test substance, immobile organisms were not removed from the test chambers at each observation time.

5.11 Statistical Analyses

The immobility data were analyzed using the computer program of C. E. Stephan (5). The program was designed to calculate the EC_{50} value and the 95% fiducial limits by probit analysis, the moving average method, and binomial probability with nonlinear interpolation (6, 7). Based on the immobility pattern in this study, binomial probability was used to calculate the 24 and 48-hour EC_{50} values. The highest nominal test concentration causing no immobility at test end and the lowest nominal test concentration causing 100% immobility at test end were assessed by visual observation of the immobility data. The no-observed-effect concentration (NOEC) based on immobility was analyzed using Cochran-Armitage (O) Trend Step-Down Test in CETIS™ v1.9.3.0 (8).

6.0 Results and Discussion

6.1 Analytical Report

Chromatographic Results

CAS # eluted as a well-resolved peak with a retention time of approximately 7.75 minutes. The LOQ was 0.400 mg a.i./L.

Test Solution Results

Test solutions in the test chambers of the 1.3, 2.5, 5.0, 10 and 20 mg a.i./L treatment groups appeared clear and colorless during the test, with no evidence of precipitation observed.

The measured concentrations of the samples ranged from below the limit of quantitation (<LOQ) to 16.5% of nominal (Table 2). The measured concentrations for the matrix fortification samples ranged from 64.4 to 100% of nominal (Table 3).

Blank control solutions showed no detected concentrations of CAS # that were greater than or equal to 30% of the method LOQ (Table 3).

A representative calibration curve is presented in Appendix 5, Figure 2. Representative chromatograms of low and high-level calibration standards are presented in Appendix 5, Figures 3 and 4. Representative chromatograms of a matrix blank sample and a matrix fortification sample are presented in Appendix 5, Figures 5 and 6, respectively. A representative chromatogram of a test sample is presented in Appendix 5, Figure 7.

6.2 In-Life Report

Measurements of temperature, dissolved oxygen and pH of the water in each test chamber are presented in Table 4. Water temperatures were within the 20 ± 1°C range established for the test. Dissolved oxygen concentrations remained ≥8.4 mg/L (≥93% of saturation) throughout the test. Measurements of pH ranged from 8.3 to 8.6. The measurements of hardness, alkalinity and specific conductance in the dilution water at test initiation were typical of EAG Laboratories-Easton well water (Table 5).

Daily observations for immobility and signs of toxicity based upon nominal concentrations during the test are presented in Table 6. All daphnids in the negative control group appeared normal throughout the test. All daphnids in the 1.3 and 2.5 mg a.i./L treatment groups also appeared normal throughout the test, with no immobile daphnids or overt signs of toxicity observed. Percent immobility in the 1.3, 2.5, 5.0, 10 and 20 mg a.i./L treatment groups at test termination was 0, 0, 95, 100 and 100%, respectively. Signs of toxicity observed among the surviving daphnids in the 5.0 mg a.i./L treatment group at test termination included lethargy. The highest nominal concentration causing no immobility at test end was determined to be 2.5 mg a.i./L. The lowest nominal concentration causing 100% immobility at test end was 10 mg a.i./L. The 24-hour EC_{50} value was 8.3 mg a.i./L. An upper 95% fiducial limit could not be calculated. At a confidence level of 95%, the binomial test shows that the EC_{50} is above 5.0 mg a.i./L (Table 7). The 48-hour EC_{50} value was 3.7 mg a.i./L with 95% fiducial limits of 2.5 and 5.0 mg a.i./L (Table 7). A graph of the concentration-response curve is included in Figure 1.

6.3 Conditions for the Validity of the Test

The test met the following criteria used to judge the validity of the test:

1) Immobility and/or signs of disease or stress in the daphnids in the control group will not exceed 10% by the end of the test. In this study, no control immobility or signs of disease or stress was observed.

2) The dissolved oxygen concentration will be ≥3 mg/L throughout the test. In this study, dissolved oxygen remained ≥8.4 mg/L throughout the test.

3) Evidence should be available to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within ±20% of the nominal test concentrations. In this study, measured concentrations ranged from <LOQ to 16.5% of nominal test concentrations. Due to the volatile nature of the test substance, the reported density was used to calculate the volume of test substance to be used in each test solution and Hamilton gas-tight syringes were used to measure the appropriate volume. Additionally, the test chambers were closed bottles with minimal headspace. The test chambers were not opened during the 48-hour exposure period.

7.0 Conclusions

The cladoceran (*Daphnia magna*) was exposed to CAS # at nominal concentrations ranging from 1.3 to 20 mg a.i./L. The 48-hour EC_{50} value, based on nominal concentrations was 3.7 mg a.i./L, with 95% fiducial limits of 2.5 and 5.0 mg a.i./L. The NOEC was 2.5 mg a.i./L. The highest nominal concentration causing no immobility at test end was determined to be 2.5 mg a.i./L. The lowest nominal concentration causing 100% immobility at test end was 10 mg a.i./L.

8.0 References

1 **Organisation for Economic Cooperation and Development.** 2004. OECD Guidelines for Testing of Chemicals, Guideline 202: *Daphnia sp., Acute Immobilization Test.* Adopted 13 April 2004.

2 **U.S. Environmental Protection Agency.** 2016. Series 850 – Ecological Effects Test Guidelines, OCSPP 850.1010: *Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids*.

3 **American Society for Testing and Materials.** 2014. ASTM Standard E 729-96: *Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians*.

4 **APHA, AWWA, WEF.** 2012. *Standard Methods for the Examination of Water and Wastewater*. 22nd Edition, American Public Health Association. American Water Works Association. Water Environment Federation. Washington, D.C.

5 **Stephan, C.E.** 1982. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

6 **Peltier, W.H. and C.I. Weber.** 1985. "Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms". United States Environmental Protection Agency. EPA/600/4-85/013. Pp. 216.

7 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

8 **Comprehensive Environmental Toxicity Information System (CETIS)**. 2000 - 2017. Version 1.9.3.0. Tidepool Scientific, LLC, McKinleyville, California.

Table 1 Immobility and Observations During a Non-GLP Range-Finding Test

Nominal Concentration [1] (mg a.i./L)	Number Immobile in 24-Hour Period / Cumulative Number Immobile / Number Originally Exposed (Observations [2])		Cumulative Percent Immobility
	24 Hours	48 Hours	
Negative Control	0 / 0 / 10 (10 AN)	0 / 0 / 10 (10 AN)	0
0.95	0 / 0 / 10 (10 AN)	0 / 0 / 10 (10 AN)	0
3.8	0 / 0 / 10 (10 AN)	0 / 0 / 10 (10 AN)	0
15	5 / 5 / 10 (5 C)	5 / 10 / 10	100
61	9 / 9 / 10 (1 C)	1 / 10 / 10	100

[1] Test solution appearance: 0.95 – 15 mg a.i./L = clear and colorless throughout the study with no evidence of precipitate; 61 mg a.i./L = slightly translucent and colorless.
[2] Observations: AN = appears normal; C = lethargic.

Table 2 Measured Concentrations of CAS # in Freshwater

Nominal Concentration (mg a.i./L)	Sample Number (783A-103A-)	Sampling Time (Days)	Measured Concentration (mg a.i./L)[1]	Percent of Nominal[1]	Mean Measured Concentration (mg a.i./L)[1]	Mean Measured Percent of Nominal
Negative	1	0	ND[2]	--	ND	--
Control	7	2	ND	--		
	8	2	ND	--		
1.3	2	0	< LOQ[3]	--	< LOQ	--
	9	2	< LOQ	--		
	10	2	< LOQ	--		
2.5	3	0	< LOQ	--	< LOQ	--
	11	2	< LOQ	--		
	12	2	< LOQ	--		
5.0	4	0	< LOQ	--	< LOQ	--
	13	2	< LOQ	--		
	14	2	< LOQ	--		
10	5	0	< LOQ	--	< LOQ	--
	15	2	< LOQ	--		
	16	2	< LOQ	--		
20	6	0	3.30	16.5	2.4 ± 1.22[4]	12.0
	17	2	1.56	7.79	CV = 50.2%	
	18	2	1.58	7.88		
		Day 2 Average	**1.57**			

[1] Results were generated using Microsoft Excel 2010 in full precision mode. Manual calculations may differ slightly.

[2] ND denotes not detected. The method limit of detection (LOD) was 0.00502 mg a.i./L.

[3] The method limit of quantitation (LOQ) for these analyses was set at 0.400 mg a.i./L, based upon the lowest analyte concentration in a fortified sample.

[4] The mean measured concentration was calculated as follows: [(Day 0 + Day 2 Average)/2].

Table 3 Quality Control Samples of CAS # in Freshwater

Sample Number (783A-103A-)	Sample Type	Concentration of CAS# Fortified (mg a.i./L)	Measured[1,3] (mg a.i./L)	Percent Recovery[3]
MAB-1	Matrix Blank	0.0	ND	--
MAB-2	Matrix Blank	0.0	< 30% of LOQ[2]	--
MAS-1	Matrix Fortification	0.400	0.258	64.4
MAS-2	Matrix Fortification	50.0	46.3	92.6
MAS-3	Matrix Fortification	0.400	0.402	100
MAS-4	Matrix Fortification	1.00	0.982	98.2
MAS-5	Matrix Fortification	50.0	44.5	89.0
			Mean[3] =	88.8
			SD[3] =	14.3
			RSD[3] =	16.2%

[1] ND denotes not detected. The method limit of detection (LOD) was 0.00502 mg a.i./L.

[2] The method limit of quantitation (LOQ) for these analyses was set at 0.400 mg a.i./L, based upon the lowest analyte concentration in a fortified sample. The analyte was detected in this sample at less than 30% of the method LOQ.

[3] Results were generated using Microsoft Excel 2010 in full precision mode. Manual calculations may differ slightly.

Table 4 Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Nominal Concentration (mg a.i./L)	Rep.	0 Hours			48 Hours		
		Temp. [1] (ºC)	DO [2] (mg/L)	pH	Temp. [1] (ºC)	DO [2] (mg/L)	pH
Negative Control	A	20.1	8.7	8.4	20.4	8.4	8.3
	B	20.1	8.7	8.5	20.4	8.5	8.4
	C	20.0	8.7	8.5	20.4	8.6	8.4
	D	20.0	8.7	8.5	20.5	8.6	8.5
1.3	A	19.9	8.7	8.5	20.5	8.7	8.5
	B	19.9	8.6	8.5	20.5	8.7	8.5
	C	19.9	8.6	8.5	20.4	8.7	8.5
	D	19.9	8.6	8.5	20.4	8.8	8.5
2.5	A	20.0	8.6	8.5	20.3	8.5	8.5
	B	20.0	8.6	8.6	20.3	8.5	8.4
	C	20.0	8.6	8.5	20.4	8.6	8.5
	D	20.0	8.6	8.6	20.4	8.4	8.5
5.0	A	20.1	8.7	8.6	20.4	8.4	8.5
	B	20.2	8.7	8.4	20.4	8.5	8.4
	C	20.2	8.7	8.4	20.4	8.5	8.4
	D	20.2	8.7	8.4	20.4	8.5	8.4
10	A	20.3	8.5	8.5	20.4	8.5	8.4
	B	20.4	8.5	8.6	20.4	8.5	8.4
	C	20.4	8.4	8.6	20.4	8.5	8.4
	D	20.5	8.4	8.6	20.4	8.4	8.4
20	A	21.0	8.5	8.6	20.4	8.4	8.5
	B	21.0	8.5	8.6	20.4	8.4	8.5
	C	21.0	8.4	8.6	20.4	8.4	8.5
	D	21.0	8.4	8.6	20.4	8.4	8.5

[1] Manual temperature measurements. Temperature monitored continuously during the test ranged from 20.10 to 20.28ºC, measured to the nearest 0.01ºC.

[2] A dissolved oxygen concentration of 5.4 mg/L represents 60% saturation at 20.0ºC in freshwater. A dissolved oxygen concentration of 9.1 mg/L represents 100% saturation at 20.0ºC in freshwater.

Table 5 Specific Conductance, Hardness and Alkalinity Measured in Well Water at Test Initiation

Parameter	Day 0
Specific Conductance (µS/cm)	312
Hardness (mg/L as $CaCO_3$)	148
Alkalinity (mg/L as $CaCO_3$)	180

Table 6 Immobility and Observations

Nominal Concentration (mg a.i./L)	Rep.	No. Exposed	~3.5 Hours		24 Hours		48 Hours		Cumulative Percent Immobility
			Number Immobile[1]	Effects[2]	Number Immobile[1]	Effects[2]	Number Immobile[1]	Effects[2]	
Negative Control	A	5	0	5 AN	0	5 AN	0	5 AN	0
	B	5	0	5 AN	0	5 AN	0	5 AN	
	C	5	0	5 AN	0	5 AN	0	5 AN	
	D	5	0	5 AN	0	5 AN	0	5 AN	
1.3	A	5	0	5 AN	0	5 AN	0	5 AN	0
	B	5	0	5 AN	0	5 AN	0	5 AN	
	C	5	0	5 AN	0	5 AN	0	5 AN	
	D	5	0	5 AN	0	5 AN	0	5 AN	
2.5	A	5	0	5 AN	0	5 AN	0	5 AN	0
	B	5	0	5 AN	0	5 AN	0	5 AN	
	C	5	0	5 AN	0	5 AN	0	5 AN	
	D	5	0	5 AN	0	5 AN	0	5 AN	
5.0	A	5	0	5 AN	0	5 AN	5	--	95
	B	5	0	5 AN	0	5 AN	5	--	
	C	5	0	5 AN	0	5 AN	4	1 C	
	D	5	0	5 AN	0	2 C; 3 AN	5	--	

[1] Cumulative number of immobile daphnia.
[2] Observed effects: AN = appear normal; C = lethargic.

Table 6 Immobility and Observations (continued)

Nominal Concentration (mg a.i./L)	Rep.	No. Exposed	~3.5 Hours		24 Hours		48 Hours		Cumulative Percent Immobility
			Number Immobile[1]	Effects[2]	Number Immobile[1]	Effects[2]	Number Immobile[1]	Effects[2]	
10	A	5	0	5 AN	3	1 C; 1 AN	5	--	100
	B	5	0	5 AN	4	1 AN	5	--	
	C	5	0	5 AN	4	1 AN	5	--	
	D	5	0	5 AN	4	1 AN	5	--	
20	A	5	0	5 AN	4	1 AN	5	--	100
	B	5	0	5 AN	3	1 C; 1 AN	5	--	
	C	5	0	5 AN	4	1 AN	5	--	
	D	5	0	5 AN	3	1 C; 1 AN	5	--	

[1] Cumulative number of immobile daphnia.
[2] Observed effects: AN = appear normal; C = lethargic.

Table 7 EC_{50} Values

Based on nominal concentrations (mg a.i./L):

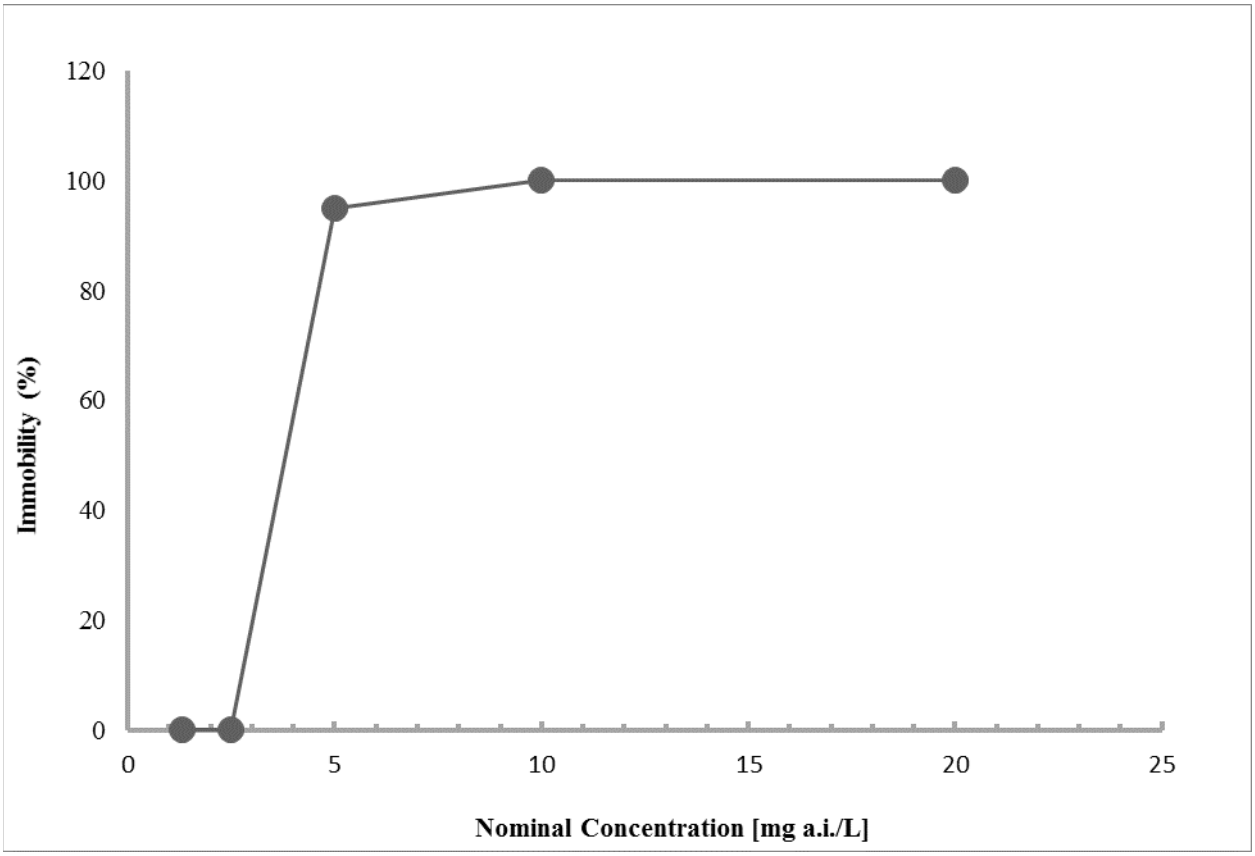
Time	EC_{50} (mg a.i./L)	95% Fiducial Limits (mg a.i./L)	Statistical Method
24 Hours	8.3	>5.0[1]	Binominal Analysis[2]
48 Hours	3.7	2.5 – 5.0	Binominal Analysis[3]

[1] An upper 95% fiducial limit could not be calculated. At a confidence level of 95%, the binomial test shows that the EC_{50} is above 5 mg a.i./L.

[2] The EC_{50} value was estimated using non-linear interpolation between 5.0 and 10 mg a.i./L; the 95% confidence limits were determined by binomial probability.

[3] The EC_{50} value was estimated using non-linear interpolation between 2.5 and 5.0 mg formulation/L; the 95% confidence limits were determined by binomial probability.

Figure 1 Concentration-Response Curve (48-Hour Immobility Data)



Appendix 1 Certificate of Analysis

Appendix 1 Certificate of Analysis (continued)

Appendix 2 Analyses of Pesticides, Organics and Metals in YCT

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Analysis Report

Sample Description: YCT
Grab Liquid INV# 17-40
2017 Facility Samples

Project Name: 2017 Facility Samples

Submittal Date/Time: 12/27/2017 09:15
Collection Date/Time: 12/19/2017

Wildlife International,
ELLE Sample #: G5 9386482
ELLE Group #: 1891009
Matrix: Liquid

CAT No.	Analysis Name	CAS Number	Dry Result		Dry Limit of Quantitation	Dilution Factor
PCBs	**SW-846 8082**		ug/kg		ug/kg	
10736	PCB-1016	12674-11-2	< 33,000	D1	33,000	1
10736	PCB-1221	11104-28-2	< 33,000	D1	33,000	1
10736	PCB-1232	11141-16-5	< 33,000	D1	33,000	1
10736	PCB-1242	53469-21-9	< 33,000	D1	33,000	1
10736	PCB-1248	12672-29-6	< 33,000	D1	33,000	1
10736	PCB-1254	11097-69-1	< 33,000	D1	33,000	1
10736	PCB-1260	11096-82-5	< 33,000	D1	33,000	1
Pesticides	**SW-846 8081A**		ug/kg		ug/kg	
14586	Aldrin	309-00-2	< 8,100	D1	8,100	5
14586	Alpha BHC	319-84-6	< 8,100	D1	8,100	5
14586	Beta BHC	319-85-7	< 9,800	D1	9,800	5
14586	Gamma BHC - Lindane	58-89-9	< 8,100	D1	8,100	5
14586	Alpha Chlordane	5103-71-9	< 8,100	D1	8,100	5
14586	Chlordane	57-74-9	< 170,000	D2	170,000	5
14586	Gamma Chlordane	5103-74-2	< 8,100	D1	8,100	5
14586	o,p-DDD	53-19-0	< 17,000	D1	17,000	5
14586	p,p-DDD	72-54-8	< 17,000	D2	17,000	5
14586	o,p-DDE	3424-82-6	< 17,000	D1	17,000	5
14586	p,p-DDE	72-55-9	< 17,000	D2	17,000	5
14586	o,p-DDT	789-02-6	< 17,000	D1	17,000	5
14586	p,p-DDT	50-29-3	< 17,000	D1	17,000	5
14586	Delta BHC	319-86-8	< 8,800	D1	8,800	5
14586	Dieldrin	60-57-1	< 17,000	D1	17,000	5
14586	Endosulfan I	959-98-8	< 8,100	D1	8,100	5
14586	Endosulfan II	33213-65-9	< 17,000	D1	17,000	5
14586	Endosulfan Sulfate	1031-07-8	< 17,000	D1	17,000	5
14586	Endrin	72-20-8	< 17,000	D1	17,000	5
14586	Endrin Aldehyde	7421-93-4	< 17,000	D1	17,000	5
14586	Endrin Ketone	53494-70-5	< 18,000	D1	18,000	5
14586	HCB	118-74-1	< 8,100	D1	8,100	5
14586	Heptachlor	76-44-8	< 8,100	D1	8,100	5
14586	Heptachlor Epoxide	1024-57-3	< 8,100	D1	8,100	5
14586	Kepone	143-50-0	< 69,000	ZD1	69,000	5
	The QC window for kepone is advisory due to the erratic performance of the analyte using this method.					
14586	Methoxychlor	72-43-5	< 66,000	ZD1	66,000	5
14586	Mirex	2385-85-5	< 17,000	D1	17,000	5
14586	Telodrin	297-78-9	< 12,000	D1	12,000	5
14586	Toxaphene	8001-35-2	< 320,000	D2	320,000	5

Z=The % difference for the calibration verification standard is outside the +/- 15% criteria. Since the average of the% difference values meets the criteria, the results are reported.

Reporting limits were raised due to interference from the sample matrix.

Pesticides	**SW-846 8141A**		ug/kg		ug/kg	
10408	Bolstar	35400-43-2	< 66,000	D1	66,000	1
10408	Coumaphos	56-72-4	< 66,000	ZD1	66,000	1
10408	Demeton-O	298-03-3	< 66,000	D1	66,000	1

Appendix 2 Analyses of Pesticides, Organics and Metals in YCT (Continued)

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Analysis Report

Sample Description: YCT
Grab Liquid INV# 17-40
2017 Facility Samples

Project Name: 2017 Facility Samples

Submittal Date/Time: 12/27/2017 09:15
Collection Date/Time: 12/19/2017

Wildlife International,
ELLE Sample #: G5 9386482
ELLE Group #: 1891009
Matrix: Liquid

CAT No.	Analysis Name	CAS Number	Dry Result		Dry Limit of Quantitation	Dilution Factor
Pesticides	**SW-846 8141A**		ug/kg		ug/kg	
10408	Demeton-S	126-75-0	< 66,000	D1	66,000	1
10408	Diazinon	333-41-5	< 66,000	D2	66,000	1
10408	Dichlorvos	62-73-7	< 66,000	D2	66,000	1
13178	Dimethoate	60-51-5	< 130,000	D2	130,000	1
10408	Disulfoton	298-04-4	< 66,000	D1	66,000	1
13178	Disulfoton	298-04-4	< 130,000	D2	130,000	1
10408	Dursban (Chlorpyrifos)	2921-88-2	< 66,000	D1	66,000	1
10408	EPN	2104-64-5	< 66,000	D1	66,000	1
10408	Ethion	563-12-2	< 66,000	D1	66,000	1
10408	Ethoprop	13194-48-4	< 66,000	D2	66,000	1
10408	Ethyl Parathion	56-38-2	< 66,000	D1	66,000	1
13178	Ethyl Parathion	56-38-2	< 130,000	D1	130,000	1
10408	Famphur	52-85-7	< 66,000	ZD1	66,000	1
13178	Famphur	52-85-7	< 130,000	D1	130,000	1
10408	Fensulfothion	115-90-2	< 200,000	D1	200,000	1
10408	Fenthion	55-38-9	< 66,000	D1	66,000	1
10408	Guthion (Azinphos-methyl)	86-50-0	< 66,000	ZD1	66,000	1
10408	Malathion	121-75-5	< 66,000	D1	66,000	1
10408	Merphos	150-50-5	< 66,000	D1	66,000	1
10408	Methyl Parathion	298-00-0	< 66,000	D1	66,000	1
13178	Methyl Parathion	298-00-0	< 130,000	D1	130,000	1
10408	Mevinphos	7786-34-7	< 66,000	D1	66,000	1
10408	Naled	300-76-5	< 66,000	ZD1	66,000	1
10408	Phorate	298-02-2	< 66,000	D1	66,000	1
13178	Phorate	298-02-2	< 130,000	D1	130,000	1
10408	Ronnel	299-84-3	< 66,000	D1	66,000	1
10408	Stirofos	961-11-5	< 66,000	D1	66,000	1
13178	Sulfotepp	3689-24-5	< 130,000	D1	130,000	1
13178	Thionazin	297-97-2	< 130,000	D1	130,000	1
10408	Tokuthion	34643-46-4	< 66,000	D1	66,000	1
10408	Trichloronate	327-98-0	< 66,000	D1	66,000	1
10408	Trithion	786-19-6	< 66,000	D1	66,000	1

The response for a target analyte(s) in the continuing calibration verification standard is outside the QC acceptance limits. Since the response is high indicating increased sensitivity, and the target analyte(s) is not detected in the sample, the data is reported.

Z=The response for a target analyte(s) in the continuing calibration verification standard is outside the QC acceptance limits. The response is low. The client was contacted and the data reported.

The recovery for the method blank surrogate(s) is outside the QC acceptance limits as noted on the QC Summary. The client was contacted and the data reported.

The recovery for a target analyte(s) in the Laboratory Control Spike(s) is outside the QC acceptance limits as noted on the QC Summary. The client was contacted and the data reported.

Appendix 2 Analyses of Pesticides, Organics and Metals in YCT (Continued)

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Analysis Report

Sample Description: YCT
Grab Liquid INV# 17-40
2017 Facility Samples

Project Name: 2017 Facility Samples

Submittal Date/Time: 12/27/2017 09:15
Collection Date/Time: 12/19/2017

Wildlife International,
ELLE Sample #: G5 9386482
ELLE Group #: 1891009
Matrix: Liquid

CAT No.	Analysis Name	CAS Number	Dry Result	Dry Limit of Quantitation	Dilution Factor
Metals		**SW-846 6010B**	mg/kg	mg/kg	
01643	Aluminum	7429-90-5	< 20,000	20,000	1
06944	Antimony	7440-36-0	< 2,000	2,000	1
06935	Arsenic	7440-38-2	< 2,000	2,000	1
06946	Barium	7440-39-3	< 500	500	1
06947	Beryllium	7440-41-7	< 500	500	1
06949	Cadmium	7440-43-9	< 500	500	1
01650	Calcium	7440-70-2	< 20,000	20,000	1
06951	Chromium	7440-47-3	< 1,500	1,500	1
06952	Cobalt	7440-48-4	< 500	500	1
06953	Copper	7440-50-8	< 1,000	1,000	1
01654	Iron	7439-89-6	< 20,000	20,000	1
06955	Lead	7439-92-1	< 1,500	1,500	1
01657	Magnesium	7439-95-4	< 10,000	10,000	1
06958	Manganese	7439-96-5	< 495	495	1
06961	Nickel	7440-02-0	< 1,000	1,000	1
01662	Potassium	7440-09-7	< 50,000	50,000	1
06936	Selenium	7782-49-2	< 2,000	2,000	1
06966	Silver	7440-22-4	< 500	500	1
01667	Sodium	7440-23-5	< 100,000	100,000	1
06925	Thallium	7440-28-0	< 3,000	3,000	1
06971	Vanadium	7440-62-2	< 500	500	1
06972	Zinc	7440-66-6	< 2,000	2,000	1
		SW-846 7471A	mg/kg	mg/kg	
00159	Mercury	7439-97-6	< 19,400	19,400	200
	Reporting limits for mercury were raised due to interference from the sample matrix.				
Wet Chemistry		**EPA 300.0**	mg/kg	mg/kg	
07335	Bromide by IC (solid)	24959-67-9	< 4,970	4,970	1
07333	Chloride by IC (solid)	16887-00-6	19,700	9,940	1
07332	Fluoride by IC (solid)	16984-48-8	1,390	994	1
07336	Nitrate Nitrogen by IC (solid)	14797-55-8	< 1,490	1,490	1
07334	Nitrite Nitrogen by IC (solid)	14797-65-0	< 994	994	1
07338	Sulfate by IC (solid)	14808-79-8	15,100	14,900	1
Wet Chemistry		**SM 2540 G-1997 %Moisture Calc**	%	%	
00111	Moisture	n.a.	99.9	0.50	1
	Moisture represents the loss in weight of the sample after oven drying at 103 - 105 degrees Celsius. The moisture result reported is on an as-received basis.				

Sample Comments

All QC is compliant unless otherwise noted. Please refer to the Quality Control Summary for overall QC performance data and associated samples.

Appendix 2 Analyses of Pesticides, Organics and Metals in YCT (Continued)

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Analysis Report

Sample Description: YCT
Grab Liquid INV# 17-40
2017 Facility Samples

Project Name: 2017 Facility Samples

Submittal Date/Time: 12/27/2017 09:15
Collection Date/Time: 12/19/2017

Wildlife International,
ELLE Sample #: G5 9386482
ELLE Group #: 1891009
Matrix: Liquid

Laboratory Sample Analysis Record

CAT No.	Analysis Name	Method	Trial#	Batch#	Analysis Date and Time	Analyst	Dilution Factor
10736	PCBs in Soil (microwave)	SW-846 8082	1	180020018A	01/04/2018 06:45	Kirby B Turner	1
14586	Pesticides in Soil	SW-846 8081A	1	180020015A	01/24/2018 17:34	Andrea L Jones	5
13178	OP Pest (APP IX), soil	SW-846 8141A	1	180020032A	01/25/2018 22:14	Richard A Shober	1
10408	OP Pest soils 8141A Master	SW-846 8141A	1	173630018A	01/11/2018 14:10	Richard A Shober	1
13181	OP Pest Solid Ext. for APP IX	SW-846 3540C	1	180020032A	01/02/2018 22:05	Karen L Beyer	1
06677	OP Pesticides Solid Extraction	SW-846 3540C	1	173630018A	01/02/2018 10:45	David S Schrum	1
10497	PCB Microwave Soil Extraction	SW-846 3546	1	180020018A	01/02/2018 23:45	Sherry L Morrow	1
10496	PPL Pest. Microwave Extraction	SW-846 3546	1	180020015A	01/02/2018 23:45	Sherry L Morrow	1
01643	Aluminum	SW-846 6010B	1	180050570801	01/10/2018 11:41	Eric L Eby	1
06944	Antimony	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06935	Arsenic	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06946	Barium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06947	Beryllium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06949	Cadmium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
01650	Calcium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06951	Chromium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06952	Cobalt	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06953	Copper	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
01654	Iron	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06955	Lead	SW-846 6010B	1	180050570801	01/10/2018 11:41	Eric L Eby	1
01657	Magnesium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06958	Manganese	SW-846 6010B	1	180110570801	01/12/2018 09:45	Suzanne M Will	1
06961	Nickel	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
01662	Potassium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06936	Selenium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06966	Silver	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
01667	Sodium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06925	Thallium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06971	Vanadium	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
06972	Zinc	SW-846 6010B	1	180050570801	01/09/2018 21:16	Elaine F Stoltzfus	1
00159	Mercury	SW-846 7471A	1	180080571101	01/10/2018 07:25	Damary Valentin	200
05708	ICP-ICPMS - SW, 3050B - U3	SW-846 3050B	1	180050570801	01/07/2018 23:39	Denise L Trimby	1
05708	ICP-ICPMS - SW, 3050B - U3	SW-846 3050B	2	180110570801	01/11/2018 23:29	Denise L Trimby	1
05711	Hg-SW, 7471A - U3	SW-846 7471A	1	180080571101	01/09/2018 16:55	JoElla L Rice	1
07335	Bromide by IC (solid)	EPA 300.0	2	17363363201A	12/30/2017 08:57	Clinton M Wilson	1
07333	Chloride by IC (solid)	EPA 300.0	1	17363363201A	01/04/2018 03:01	Clinton M Wilson	1
07332	Fluoride by IC (solid)	EPA 300.0	2	17363363201A	12/30/2017 08:57	Clinton M Wilson	1
07336	Nitrate Nitrogen by IC (solid)	EPA 300.0	2	17363363201A	12/30/2017 08:57	Clinton M Wilson	1
07334	Nitrite Nitrogen by IC (solid)	EPA 300.0	2	17363363201A	12/30/2017 08:57	Clinton M Wilson	1
07338	Sulfate by IC (solid)	EPA 300.0	2	17363363201A	12/30/2017 08:57	Clinton M Wilson	1
01352	Deionized Water Extraction	EPA 300.0	1	17363363201A	12/29/2017 15:00	Luke Timcik	1
00111	Moisture	SM 2540 G-1997 %Moisture Calc	1	18002820005B	01/03/2018 14:48	Larry E Bevins	1

Appendix 2 Analyses of Pesticides, Organics and Metals in YCT (Continued)

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Data Qualifiers

Qualifier	Definition
C	Result confirmed by reanalysis
D1	Indicates for dual column analyses that the result is reported from column 1
D2	Indicates for dual column analyses that the result is reported from column 2
E	Concentration exceeds the calibration range
J (or G, I, X)	Estimated value >= the Method Detection Limit (MDL or DL) and < the Limit of Quantitation (LOQ or RL)
P	Concentration difference between the primary and confirmation column >40%. The lower result is reported.
U	Analyte was not detected at the value indicated
V	Concentration difference between the primary and confirmation column >100%. The reporting limit is raised due to this disparity and evident interference.
W	The dissolved oxygen uptake for the unseeded blank is greater than 0.20 mg/L.
Z	Laboratory Defined - see analysis report

Additional Organic and Inorganic CLP qualifiers may be used with Form 1 reports as defined by the CLP methods.
Qualifiers specific to Dioxin/Furans and PCB Congeners are detailed on the individual Analysis Report.

Appendix 3 Specific Conductance, Hardness, Alkalinity, pH and Total Organic Carbon (TOC) of Well Water Measured During the Approximate 4-Week Period Immediately Preceding the Test

Parameter	Mean	Range
Specific Conductance (µS/cm)	329 (N=4)	311 – 338
Hardness (mg/L as $CaCO_3$)	137 (N=4)	132 – 140
Alkalinity (mg/L as $CaCO_3$)	177 (N=4)	176 – 178
pH	8.0 (N=4)	7.8 – 8.2
Total Organic Carbon (TOC) (mg C/L)	<1 (N = 1) [1]	-- [1]

[1] TOC is measured monthly.

Appendix 4 Analyses of Pesticides, Organics and Metals in EAG Laboratories-Easton Well Water

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Analysis Report

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Sample Description: Well Water
2017 Facility Samples

Project Name: 2017 Facility Samples

Submittal Date/Time: 12/21/2017 11:55
Collection Date/Time: 12/20/2017

Wildlife International,
ELLE Sample #: WW 9381758
ELLE Group #: 1889983
Matrix: Wastewater

CAT No.	Analysis Name	CAS Number	Result	Limit of Quantitation	Dilution Factor
Pesticides	**SW-846 8081A**		ug/l	ug/l	
00177	Aldrin	309-00-2	< 0.0092 D1	0.0092	1
00177	Alpha BHC	319-84-6	< 0.0092 D2	0.0092	1
00177	Beta BHC	319-85-7	< 0.0092 D2	0.0092	1
00177	Gamma BHC - Lindane	58-89-9	< 0.0092 D1	0.0092	1
00177	Alpha Chlordane	5103-71-9	< 0.0092 D2	0.0092	1
00177	Chlordane	57-74-9	< 0.46 D2	0.46	1
00177	Gamma Chlordane	5103-74-2	< 0.018 D1	0.018	1
00177	o,p-DDD	53-19-0	< 0.018 D1	0.018	1
00177	p,p-DDD	72-54-8	< 0.018 D1	0.018	1
00177	o,p-DDE	3424-82-6	< 0.018 D1	0.018	1
00177	p,p-DDE	72-55-9	< 0.018 D1	0.018	1
00177	o,p-DDT	789-02-6	< 0.018 D1	0.018	1
00177	p,p-DDT	50-29-3	< 0.018 D1	0.018	1
00177	Delta BHC	319-86-8	< 0.0092 D2	0.0092	1
00177	Dieldrin	60-57-1	< 0.018 D1	0.018	1
00177	Endosulfan I	959-98-8	< 0.0092 D2	0.0092	1
00177	Endosulfan II	33213-65-9	< 0.028 D1	0.028	1
00177	Endosulfan Sulfate	1031-07-8	< 0.018 D1	0.018	1
00177	Endrin	72-20-8	< 0.018 D1	0.018	1
00177	Endrin Aldehyde	7421-93-4	< 0.092 D1	0.092	1
00177	Endrin Ketone	53494-70-5	< 0.018 D1	0.018	1
00177	HCB	118-74-1	< 0.0092 D1	0.0092	1
00177	Heptachlor	76-44-8	< 0.0092 D1	0.0092	1
00177	Heptachlor Epoxide	1024-57-3	< 0.0092 D1	0.0092	1
00177	Kepone	143-50-0	< 0.18 ZD1	0.18	1
00177	Methoxychlor	72-43-5	< 0.092 D1	0.092	1
00177	Mirex	2385-85-5	< 0.046 D2	0.046	1
00177	Telodrin	297-78-9	< 0.0092 D1	0.0092	1
00177	Toxaphene	8001-35-2	< 0.92 D1	0.92	1

Z=The % difference for the calibration verification standard is outside the +/- 15% criteria. Since the average of the% difference values meets the criteria, the results are reported.

Pesticides	**SW-846 8141A**		ug/l	ug/l	
10410	Bolstar	35400-43-2	< 5.2 D1	5.2	1
10410	Coumaphos	56-72-4	< 5.2 D1	5.2	1
10410	Demeton-O	298-03-3	< 5.2 D1	5.2	1
10410	Demeton-S	126-75-0	< 5.2 D1	5.2	1
10410	Diazinon	333-41-5	< 5.2 D1	5.2	1
10410	Dichlorvos	62-73-7	< 5.2 D1	5.2	1
10410	Disulfoton	298-04-4	< 5.2 D1	5.2	1
10410	Dursban (Chlorpyrifos)	2921-88-2	< 5.2 D1	5.2	1
10410	EPN	2104-64-5	< 5.2 D1	5.2	1
10410	Ethion	563-12-2	< 5.2 D1	5.2	1
10410	Ethoprop	13194-48-4	< 5.2 D1	5.2	1
10410	Ethyl Parathion	56-38-2	< 5.2 D1	5.2	1
10410	Famphur	52-85-7	< 5.2 D1	5.2	1
10410	Fensulfothion	115-90-2	< 7.9 D1	7.9	1
10410	Fenthion	55-38-9	< 5.2 D1	5.2	1
10410	Guthion (Azinphos-methyl)	86-50-0	< 5.2 D1	5.2	1
10410	Malathion	121-75-5	< 5.2 D1	5.2	1

Appendix 4 Analyses of Pesticides, Organics and Metals in EAG Laboratories-Easton Well Water (Continued)

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Analysis Report

Sample Description: Well Water
2017 Facility Samples

Project Name: 2017 Facility Samples

Submittal Date/Time: 12/21/2017 11:55
Collection Date/Time: 12/20/2017

Wildlife International,
ELLE Sample #: WW 9381758
ELLE Group #: 1889983
Matrix: Wastewater

CAT No.	Analysis Name	CAS Number	Result	Limit of Quantitation	Dilution Factor
Pesticides		**SW-846 8141A**	ug/l	ug/l	
10410	Merphos	150-50-5	< 5.2 D1	5.2	1
10410	Methyl Parathion	298-00-0	< 5.2 D1	5.2	1
10410	Mevinphos	7786-34-7	< 5.2 D1	5.2	1
10410	Naled	300-76-5	< 5.2 D1	5.2	1
10410	Phorate	298-02-2	< 5.2 D1	5.2	1
10410	Ronnel	299-84-3	< 5.2 D1	5.2	1
10410	Stirofos	961-11-5	< 5.2 D1	5.2	1
10410	Tokuthion	34643-46-4	< 5.2 D1	5.2	1
10410	Trichloronate	327-98-0	< 5.2 D1	5.2	1
10410	Trithion	786-19-6	< 5.2 D1	5.2	1
Metals		**EPA 200.7 rev 4.4**	mg/l	mg/l	
01743	Aluminum	7429-90-5	< 0.200	0.200	1
07044	Antimony	7440-36-0	< 0.0200	0.0200	1
07035	Arsenic	7440-38-2	< 0.0200	0.0200	1
07046	Barium	7440-39-3	< 0.0050	0.0050	1
07047	Beryllium	7440-41-7	< 0.0050	0.0050	1
07049	Cadmium	7440-43-9	< 0.0050	0.0050	1
01750	Calcium	7440-70-2	36.2	0.200	1
07051	Chromium	7440-47-3	< 0.0150	0.0150	1
07052	Cobalt	7440-48-4	< 0.0050	0.0050	1
07053	Copper	7440-50-8	< 0.0100	0.0100	1
01754	Iron	7439-89-6	< 0.200	0.200	1
07055	Lead	7439-92-1	< 0.0150	0.0150	1
01757	Magnesium	7439-95-4	14.0	0.100	1
07058	Manganese	7439-96-5	< 0.0050	0.0050	1
07061	Nickel	7440-02-0	< 0.0100	0.0100	1
01762	Potassium	7440-09-7	7.06	0.500	1
07036	Selenium	7782-49-2	< 0.0200	0.0200	1
07066	Silver	7440-22-4	< 0.0050	0.0050	1
01767	Sodium	7440-23-5	18.1	1.00	1
07022	Thallium	7440-28-0	< 0.0300	0.0300	1
07071	Vanadium	7440-62-2	< 0.0050	0.0050	1
07072	Zinc	7440-66-6	< 0.0200	0.0200	1
		EPA 245.1 rev 3	mg/l	mg/l	
00259	Mercury	7439-97-6	< 0.00020	0.00020	1
Wet Chemistry		**EPA 300.0**	mg/l	mg/l	
01505	Bromide	24959-67-9	< 2.5	2.5	5
00224	Chloride	16887-00-6	4.3	2.0	5
01504	Fluoride	16984-48-8	< 0.50	0.50	5
00368	Nitrate Nitrogen	14797-55-8	< 0.50	0.50	5
01506	Nitrite Nitrogen	14797-65-0	< 0.50	0.50	5
00228	Sulfate	14808-79-8	5.5	5.0	5

Sample Comments

All QC is compliant unless otherwise noted. Please refer to the Quality Control Summary for overall QC performance data and associated samples.

Appendix 4 Analyses of Pesticides, Organics and Metals in EAG Laboratories-Easton Well Water (Continued)

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Analysis Report

Sample Description: Well Water
2017 Facility Samples

Project Name: 2017 Facility Samples

Submittal Date/Time: 12/21/2017 11:55
Collection Date/Time: 12/20/2017

Wildlife International,
ELLE Sample #: WW 9381758
ELLE Group #: 1889983
Matrix: Wastewater

Laboratory Sample Analysis Record

CAT No.	Analysis Name	Method	Trial#	Batch#	Analysis Date and Time	Analyst	Dilution Factor
00177	CC Pesticides in Water	SW-846 8081A	1	173610004A	01/09/2018 23:08	Heather E Williams	1
10410	OP Pest water 8141A Master	SW-846 8141A	1	173610007A	01/05/2018 23:34	Richard A Shober	1
06654	OP Pest Water Ext. (Turbovap)	SW-846 3510C	1	173610007A	12/27/2017 18:54	Kate E Lutte	1
11118	Pesticide Screen Waters Ext	SW-846 3510C	1	173610004A	12/27/2017 18:54	Kate E Lutte	1
01743	Aluminum	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07044	Antimony	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07035	Arsenic	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07046	Barium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07047	Beryllium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07049	Cadmium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
01750	Calcium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07051	Chromium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07052	Cobalt	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07053	Copper	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
01754	Iron	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07055	Lead	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
01757	Magnesium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07058	Manganese	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07061	Nickel	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
01762	Potassium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07036	Selenium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07066	Silver	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
01767	Sodium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07022	Thallium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07071	Vanadium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07072	Zinc	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
00259	Mercury	EPA 245.1 rev 3	1	173560571404	12/27/2017 09:47	Damary Valentin	1
05716	EPA 600 ICP Digest (tot rec)	EPA 200.7 rev 4.4	1	173560571610	12/27/2017 07:15	Lisa J Cooke	1
05714	PW/WW Hg Digest	EPA 245.1 rev 3	1	173560571404	12/27/2017 01:05	Denise L Trimby	1
01505	Bromide	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5
00224	Chloride	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5
01504	Fluoride	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5
00368	Nitrate Nitrogen	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5
01506	Nitrite Nitrogen	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5
00228	Sulfate	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5

Appendix 4 Analyses of Pesticides, Organics and Metals in EAG Laboratories-Easton Well Water (Continued)

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Data Qualifiers

Qualifier	Definition
C	Result confirmed by reanalysis
D1	Indicates for dual column analyses that the result is reported from column 1
D2	Indicates for dual column analyses that the result is reported from column 2
E	Concentration exceeds the calibration range
J (or G, I, X)	Estimated value >= the Method Detection Limit (MDL or DL) and < the Limit of Quantitation (LOQ or RL)
P	Concentration difference between the primary and confirmation column >40%. The lower result is reported.
U	Analyte was not detected at the value indicated
V	Concentration difference between the primary and confirmation column >100%. The reporting limit is raised due to this disparity and evident interference.
W	The dissolved oxygen uptake for the unseeded blank is greater than 0.20 mg/L.
Z	Laboratory Defined - see analysis report

Additional Organic and Inorganic CLP qualifiers may be used with Form 1 reports as defined by the CLP methods.
Qualifiers specific to Dioxin/Furans and PCB Congeners are detailed on the individual Analysis Report.

Appendix 5 The Analysis of CAS # in Freshwater

Appendix 5, Figure 1 Method Outline for the Analysis of CAS # in Freshwater

METHOD OUTLINE FOR THE ANALYSIS OF CAS# IN FRESHWATER

1. Prepare calibration standards in Toluene from a stock solution prepared in acetone. Transfer aliquots of each into autosampler vials for analysis. Transfer remaining to scintillation vials with Teflon®-lined caps.

2. Pre-weigh sodium chloride for each sample into weigh boats.

3. Study samples will have the appropriate volume of toluene added when collected by aquatics laboratory. Add 1.0 g of sodium chloride to each sample before vortexing each sample for approximately 1 minute using a hand vortexer.

4. Centrifuge samples at approximately 491 RCF for approximately 5 minutes in order to separate the aqueous and organic layers. Then, carefully remove an aliquot of each upper toluene layer and transfer it to an autosampler vial for analysis. Transfer the remaining toluene layers into scintillation vials with Teflon®-lined caps.

5. Lastly, prepare quality control (QC) samples in glass scintillation vials with Teflon®-lined caps.

   a. Pre-weigh sodium chloride for each QC sample into weigh boats.

   b. Add freshwater to each vial, and remove the appropriate fortification volume using a pipettor, or equivalent.

   c. To fortify each sample, use a gas-tight syringe to add the aliquot of the appropriate stock approximately 2/3 of the way beneath the water surface. The matrix blank will be unfortified freshwater.

   d. Add toluene to each matrix fortification sample immediately after fortifying.

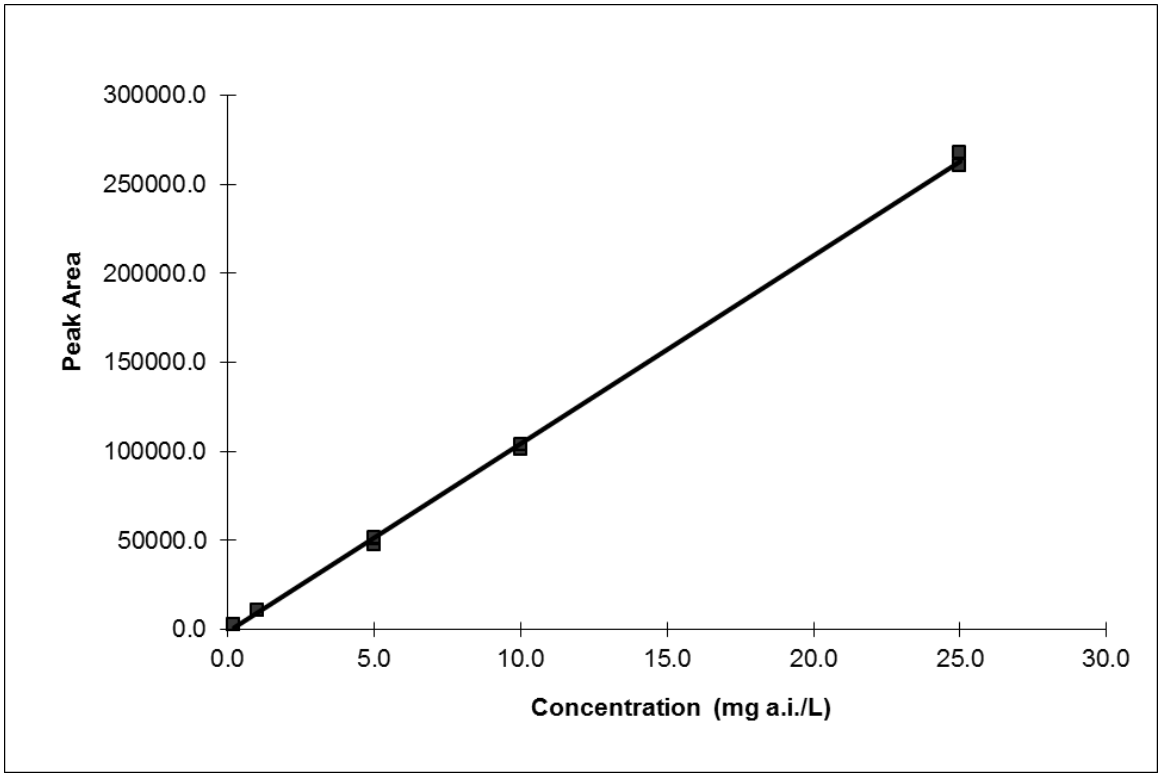
   e. Then, immediately add the pre-weighed sodium chloride, cap and invert a few times to mix.

6. Once all QC samples are prepared, vortex each sample for approximately 1 minute using a hand vortexer.

7. Centrifuge samples at approximately 491 RCF for approximately 5 minutes in order to separate the aqueous and organic layers. Then, carefully remove an aliquot of each upper toluene layer and transfer it to an autosampler vial for analysis. Transfer the remaining toluene layers into scintillation vials with Teflon®-lined caps.

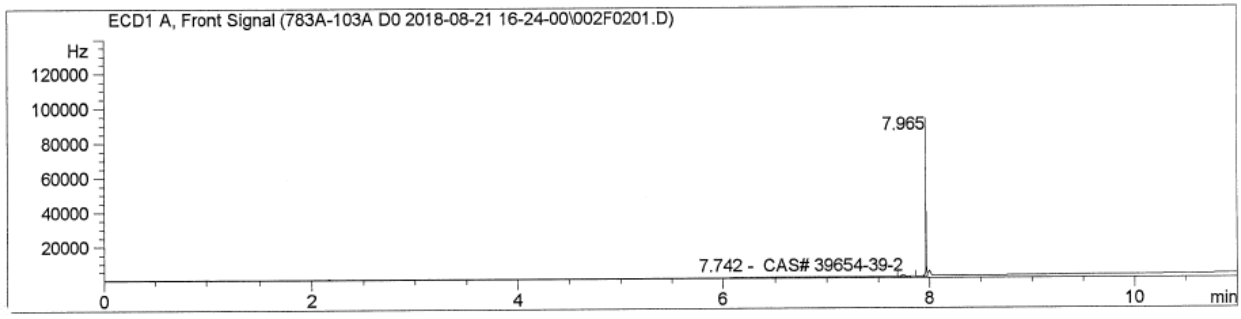
8. Submit standards and samples for analysis by GC-ECD.

Appendix 5, Figure 2 Representative Calibration Curve for CAS



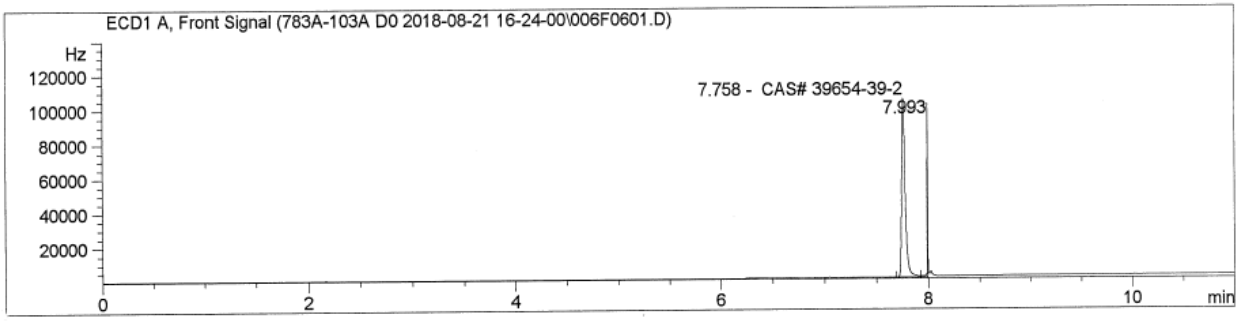
y-intercept = 365.435; Slope = 10369; R^2 = 0.99895

Appendix 5, Figure 3 Representative Chromatogram of a Low-Level Calibration Standard for CAS



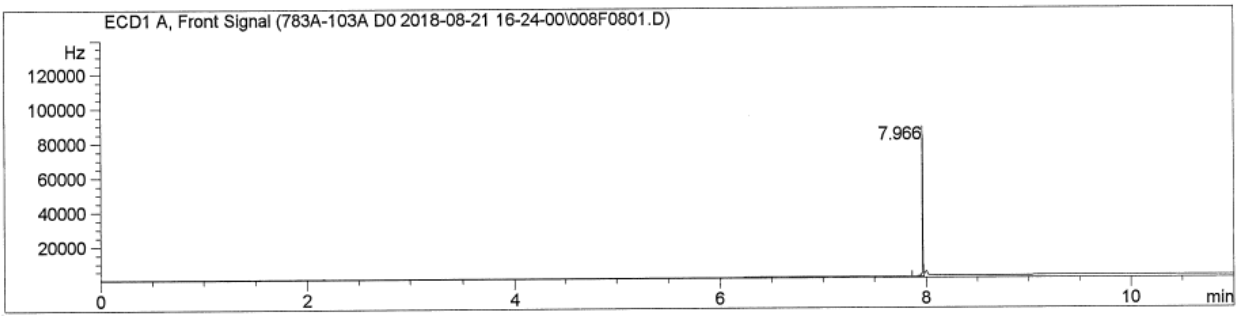
Calibration standard solution contains CAS# at a concentration of 0.200 mg a.i./L. CAS# elutes at a retention time of approximately 7.75 minutes.

Appendix 5, Figure 4 Representative Chromatogram of a High-Level Calibration Standard for CAS



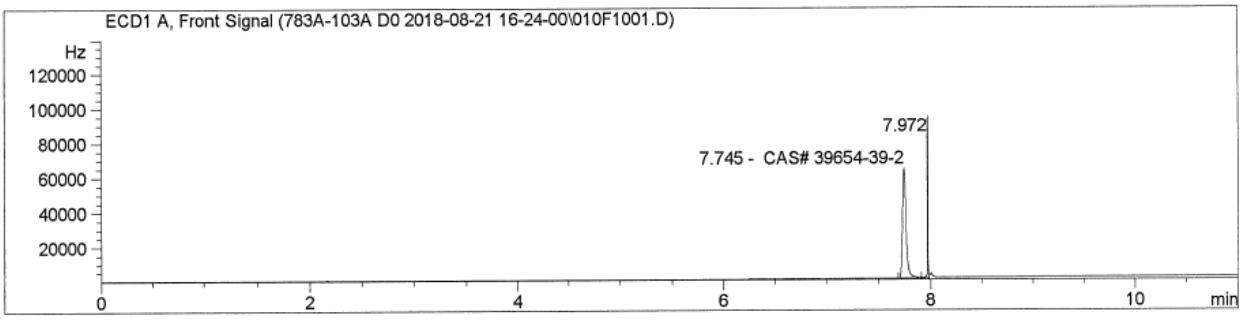
Calibration standard solution contains CAS# at a concentration of 25.0 mg a.i./L. CAS# elutes at a retention time of approximately 7.75 minutes.

Appendix 5, Figure 5 Representative Chromatogram of a Matrix Blank Sample



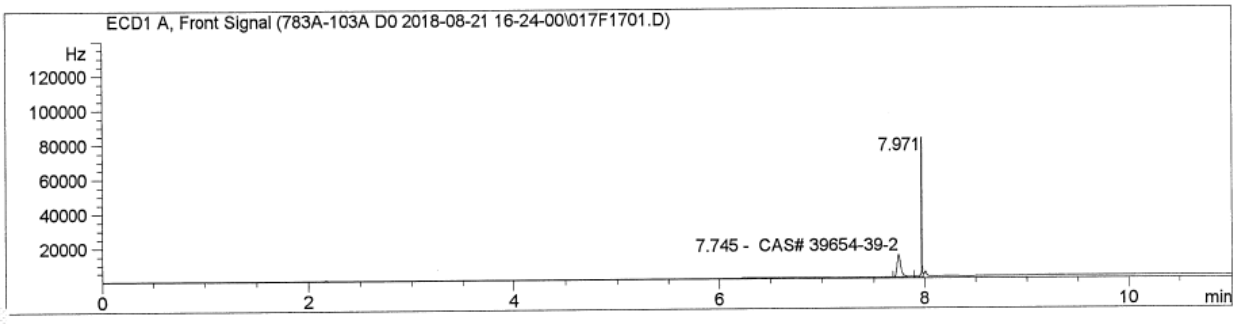
CAS# elutes at a retention time of approximately 7.75 minutes. (Sample 783A-103A-MAB-1).

Appendix 5, Figure 6 Representative Chromatogram of a Matrix Fortification Sample Analyzed for CAS



Matrix fortification sample (783A-103A-MAS-1) contains CAS# at a nominal concentration of 50.0 mg a.i./L. CAS# elutes at a retention time of approximately 7.75 minutes.

Appendix 5, Figure 7 Representative Chromatogram of a Test Sample Analyzed for CAS # in Freshwater



Test solution sample (783A-103A-6) contains CAS# at a nominal concentration of 20 mg a.i./L. CAS# elutes at a retention time of approximately 7.75 minutes.

Appendix 6 Personnel Involved in the Study

The following individuals from EAG Laboratories-Easton participated in the conduct of this study:

Study Director:	Amanda K. Gerke, B.S.
Management:	Suzanne Z. Schneider, Ph.D. Ling Zhang, Ph.D.
Supervisory Personnel:	Jessica M. Griebel, M.S.
Biologists:	Lois A. O'Boyle, M.S.
Animal Care:	Michele A. Stence, B.S.
Analysis of Test Solutions:	Laura A. Lockard, M.S.
Report Preparation:	Amanda K. Gerke, B.S. Laura A. Lockard, M.S.

Appendix 7 Changes to Protocol

This study was conducted in accordance with the approved protocol, the following exceptions:

1) Temperature, dissolved oxygen and pH were not measured at approximately 24 hours during the test.

2) Immobile daphnids were not removed from the test chambers at approximately 24 hours during the test.

3) Test concentrations were 1.3, 2.5, 5.0, 10 and 20 mg a.i./L.

FINAL REPORT

Test Facility Study No. 20146048

Sponsor Reference No.

An Acute Study of by Oral Gavage in Rat (Fixed Dose Method)

SPONSOR:

TEST FACILITY:
Charles River Laboratories Den Bosch BV
Hambakenwetering 7
5231 DD ‘s-Hertogenbosch
The Netherlands

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QUALITY ASSURANCE STATEMENT

Study title: An Acute Study of Perfluoroalkyl Vinyl Ether (n=1) by Oral Gavage in Rat (Fixed Dose Method).

This report was inspected by the Test Facility Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s). The reported method and procedures were found to describe those used and the report reflects the raw data. The Test Facility inspection program was conducted in accordance with Standard Operating Procedure. During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Test Facility Study No. 20146048

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date to TFM and SD*
Study	Final Study Plan	04-Apr-2018	04-Apr-2018	04-Apr-2018
	Report	28-Jun-2018	28-Jun-2018	28-Jun-2018
	Final Report	17-Jul-2018	17-Jul-2018	17-Jul-2018
Process	**Test Item Formulation** Test Item Handling	14-Feb-2018	28-Feb-2018	01-Mar-2018
	Necropsy Observations/Measurements Specimen Handling	05-Mar-2018	16-Mar-2018	16-Mar-2018
	Test Item Receipt Test Item Handling	13-Mar-2018	21-Mar-2018	21-Mar-2018
	Animal Facilities Test Item Handling Observations/Measurements Specimen Handling Exposure	09-Apr-2018	24-Apr-2018	24-Apr-2018

*TFM=Test Facility Management SD = Study Director

Ulrich Wiets
Quality Assurance Auditor

U Wiets

Date: 17 Jul 2018

COMPLIANCE STATEMENT AND REPORT APPROVAL

The study was performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America, Japan, and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

This study was conducted in accordance with the procedures described herein. There were no deviations from the study plan and standard operating procedures. The report represents an accurate and complete record of the results obtained. There were no deviations from the above regulations that affected the overall integrity of the study or the interpretation of the study results and conclusions.

Date: 17 Jul 2018

P.H.T. van Sas, MSc.

Study Director

1. RESPONSIBLE PERSONNEL

1.1. Test Facility

Study Director	P.H.T. van Sas, MSc
Test Facility Management	H.H. Emmen, MSc

2. SUMMARY

The objective of this study was to determine the potential toxicity of when given by oral gavage at a single dose to rats of a single sex at one or more defined doses to evaluate the potential reversibility of any findings.

The study was carried out based on the guidelines described in:

- OECD No.420 (2001) "Acute Oral Toxicity, Fixed Dose Procedure"
- EC No 440/2008, part B: "Acute Oral Toxicity, Fixed Dose Procedure"
- EPA, OPPTS 870.1100 (2002), "Acute Oral Toxicity"
- JMAFF Guidelines (2000), including the most recent revisions.

Initially, was administered by oral gavage to one female Wistar rat at 2000 mg/kg body weight. As no mortality occurred and no signs of significant toxicity were observed, the main study was conducted with a fixed dose of 2000 mg/kg body weight administered to four female rats. The animals were subjected to daily observations. Body weights were determined on Days 1, 8 and 15. Macroscopic examination was performed after terminal sacrifice.

No mortality occurred.

Hunched posture and piloerection were noted for the animals on Day 1.

The body weight gain shown by the animals over the study period was considered to be similar to that expected of normal untreated animals of the same age and strain.

No abnormalities were found at macroscopic post mortem examination of the animals.

The minimum oral lethal dose of in rats was established to exceed 2000 mg/kg body weight. Based on the absence of mortality rate at 2000 mg/kg body weight, it was concluded that the oral LD50 value of exceeds 2000 mg/kg body weight.

According to the OECD 423 test guideline, the LD50 cut-off values was considered to exceed 2000 mg/kg body weight.

Based on these results, does not have to be classified and has no obligatory labelling requirement for acute oral toxicity according to the:

- Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations (2017) (including all amendments),
- Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (including all amendments).

3. INTRODUCTION

The objective of this study was to determine the potential toxicity of when given by oral gavage at a single dose to rats of a single sex at one or more defined doses to evaluate the potential reversibility of any findings. This study is intended to provide information on the potential health hazards of and data produced can be used for classification/labelling of the test item. This study should provide a rational basis for risk assessment in man.

The design of this study is based on the following study guidelines:

- OECD Guideline 420. *Acute Oral Toxicity – Fixed Dose Procedure*, December 2001.
- EPA Health Effects Test Guideline OPPTS 870.1100. *Acute Oral Toxicity in Rodents*, December 2002.
- EC No 440/2008 Part B. *Acute Oral Toxicity*, *Fixed Dose Procedure*, May 2008.
- Appendix to Director General Notification, No. 12-Nousan-8147. Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan (JMAFF), November 2000, including the most recent revisions.

The Study Director signed the study plan on 04 Apr 2018, and dosing was initiated on 10 Apr 2018. The in-life phase of the study was completed on 01 May 2018. The experimental start date was 09 Apr 2018, and the experimental completion date was 01 May 2018. The study plan is presented in Appendix 3.

4. MATERIALS AND METHODS

4.1. Test item and Vehicle

4.1.1. Test Item

Identification:

Appearance:	Clear colourless liquid
Batch:	12639
Purity/Composition:	See Certificate of Analysis
Test item storage:	At room temperature container flushed with nitrogen
Stable under storage conditions until:	08 November 2020 (expiry date)

Additional information

Test Facility test item number:	209150/A
Purity/Composition correction factor:	No correction factor required
Test item handling:	Handle in glove box (nitrogen environment)
Chemical name (IUPAC, synonym or trade name:	

4.2. Test Item Characterization

The Sponsor provided to the Test Facility documentation of the identity, purity, composition, and stability for the test item. A Certificate of Analysis or equivalent document was provided to the Test Facility and is presented in Appendix 2.

4.3. Reserve Samples

For each batch (lot) of test item, a reserve sample (about 0.5 gram) was collected and maintained under the appropriate storage conditions by the Test Facility. The sample will be destroyed after the expiration date.

4.4. Test and Reference Item Inventory and Disposition

Records of the receipt, distribution, and storage of test item were maintained. With the exception of reserve samples, all unused Sponsor-supplied test item was discarded after completion of the scheduled program of work. Records of the decisions made will be kept at the Test Facility.

4.5. Preparation of Test Item

The Test Item, was administered as received.

Adjustment was made for specific gravity of the test item. No correction was made for the purity/composition of the test item.

Any residual volumes were discarded.

4.6. Sample Collection and Analysis

The test item was used as received from the Sponsor; therefore, samples for dose formulation analysis were not collected by the Test Facility.

4.7. Test System

Species:	Rat
Strain:	Crl: WI(Han)
Condition:	Outbred, SPF-Quality
Source:	Charles River Deutschland, Sulzfeld, Germany
Number of Animals:	5 Females. Including the single female that was dosed in the pilot study at the selected concentration. Females were nulliparous and non-pregnant.
Age at the Initiation of Dosing:	Young adult animals (approximately 9-10 weeks old) were selected.
Weight at the Initiation of Dosing:	160 to 200 g.

4.7.1. Justification for Test System and Number of Animals

The Wistar Han rat was chosen as the animal model for this study as recognized by international guidelines as a recommended test system. The test method and number of animals were based on the test guidelines.

The study plan was reviewed and agreed by the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. within the framework of project license AVD2360020172866 (Appendix 1) approved by the Central Authority for Scientific Procedures on Animals (CCD) as required by the Dutch Act on Animal Experimentation (December 2014).

4.7.2. Animal Identification

At study assignment, each animal was identified using an ear mark (not for the animals of the pilot study) and tail mark with indelible ink.

4.7.3. Environmental Acclimation

The animals were allowed to acclimate to the Test Facility toxicology accommodation for at least 5 days before the commencement of dosing.

4.7.4. Selection, Assignment, Replacement, and Disposition of Animals

Animals were assigned to the study at the discretion of the coordinating biotechnician according to body weights, with all animals within ± 20% of the sex mean. Animals in poor health or at extremes of body weight range were not assigned to the study.

Before the initiation of dosing, a health inspection was performed and any assigned animal considered unsuitable for use in the study were replaced by alternate animals obtained from the same shipment and maintained under the same environmental conditions.

The disposition of all animals was documented in the study records.

4.7.5. Husbandry

4.7.5.1. Housing

On arrival, animals were group housed (up to 5 animals of the same sex together) in polycarbonate cages (Makrolon MIV type; height 18 cm.) and following assignment to the study, animals were individually housed (pilot study) or group housed (main study, up to 5 animals of the same sex and same dosing group together) in polycarbonate cages (Makrolon MIV type; height 18 cm.) containing sterilized sawdust as bedding material (Lignocel S 8-15, JRS - J.Rettenmaier & Söhne GmbH + CO. KG, Rosenberg, Germany) equipped with water bottles. The rooms in which the animals were kept was documented in the study records.

Animals were separated during designated procedures/activities. Each cage was clearly labeled.

4.7.5.2. Environmental Conditions

Target temperatures of 18 to 24°C with a relative target humidity of 40 to 70% were maintained. The actual daily mean temperature during the study period was 21°C with an actual daily mean relative humidity of 43 to 51%. A 12-hour light/12-hour dark cycle was maintained. Ten or greater air changes per hour with 100% fresh air (no air recirculation) were maintained in the animal rooms.

4.7.5.3. Food

Pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany) was provided ad libitum throughout the study, except during designated procedures.

The feed was analyzed by the supplier for nutritional components and environmental contaminants. Results of the analysis were provided by the supplier and are on file at the Test Facility.

It is considered that there were no known contaminants in the feed that would interfere with the objectives of the study.

4.7.5.4. Water

Municipal tap-water was freely available to each animal via water bottles.

Periodic analysis of the water was performed, and results of these analyses are on file at the Test Facility.

It is considered that there were no known contaminants in the water that would interfere with the objectives of the study.

4.7.5.5. Animal Enrichment

For psychological/environmental enrichment, animals were provided with paper (Enviro-dri, Wm. Lillico & Son (Wonham Mill Ltd), Surrey, United Kingdom), except when interrupted by study procedures/activities.

4.8. Experimental Design

In order to set the dose level for the main study, a pilot study was performed in single animals. The following fixed dose levels were considered: 5, 50, 300 and 2000 mg/kg. Administration was started at 2000 mg/kg body weight in one female rat.

The main study was conducted at 2000 mg/kg, as this was the highest dose level which produced no mortality.

The methods used in the pilot study were similar to those used in the main study, except for individual housing of the pilot animals. A summary of the results is reported.

4.8.1. Administration of Test item

A single dose of test item was administered to the appropriate animals by oral gavage on Day 1, using a syringe with a plastic gavage cannula attached. The Starting dose level was 2000 mg/kg body weight.

The dose volume for each animal was based on the body weight measurement prior to dosing. Dose volume (mL/kg body weight) was calculated as follows:
Dose level (g/kg) / spec.gravity or density (g/mL).

The dosing formulations were stirred continuously during dose administration.

Animals were deprived of food overnight (for a maximum of 20 hours) prior to dosing and until 3-4 hours after administration of the test item. Water was available.

4.8.2. Justification of Route and Dose Levels

The oral route was selected as it is a possible route of human exposure during manufacture, handling or use of the test item.

The dose level was based on the results of the pilot study and was specified and approved by the Study Director in the study files. If at the selected dose level no evident toxicity was seen or mortality occured, a next higher or lower dose level was tested, respectively. The highest dose level was 2000 mg/kg body weight.

4.9. In-life Procedures, Observations, and Measurements

4.9.1. Mortality/Moribundity Checks

Throughout the study, animals were observed for general health/mortality and moribundity twice daily, in the morning and at the end of the working day. Animals were not removed from cage during observation, unless necessary for identification or confirmation of possible findings.

4.9.2. Clinical Observations

4.9.2.1. Postdose Observations

Postdose observations were performed at periodic intervals on the day of dosing (at least three times) and once daily thereafter. The observation period was 14 days.

All the animals were examined for reaction to dosing. The onset, intensity and duration of these signs was recorded (if appropriate), particular attention being paid to the animals during and for the first hour after dosing.

4.9.3. Body Weights

Animals were weighed individually on Day 1 (predose), 8 and 15.

4.10. Terminal Procedures

All animals were sacrificed by oxygen/carbon dioxide procedure at the end of the observation period. All animals assigned to the study were subjected to necropsy and descriptions of all internal macroscopic abnormalities were recorded.

5. ANALYSIS

All results presented in the tables of the report are calculated using values as per the raw data rounding procedure and may not be exactly reproduced from the individual data presented.

The oral LD50 value of the test item was ranked within the following ranges: 0-5, 5-50, 50-300 or 300-2000 mg/kg b.w. or as exceeding 2000 mg/kg b.w. The LD50 cut-off value was established based on OECD guideline 423. No statistical analysis was performed (The method used is not intended to allow the calculation of a precise LD50 value).

The results were evaluated according to:

- Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations (2017) (including all amendments).
- Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of items and mixtures (including all amendments).

6. COMPUTERIZED SYSTEMS

Critical computerized systems used in the study are listed below. All computerized systems used in the conduct of this study have been validated; when a particular system has not satisfied all requirements, appropriate administrative and procedural controls were implemented to assure the quality and integrity of data.

Text Table 1
Critical Computerized Systems

System name	Version No.	Description of Data Collected and/or Analyzed
REES Centron	SQL 2.0	Temperature and Humidity (Animal and Laboratory facilities) Data Collection
ToxData[1]	8.0	In-life phase (mortality; clinical signs; body weights) data collection

1. For logistic reasons, data was captured under study number 520768. All data was reported under Test Facility Study No. 20146048.

7. RETENTION OF RECORDS

All study-specific raw data, documentation, study plan and final report from this study were archived at the Test Facility by no later than the date of final report issue. At least five years after issue of the final report, the Sponsor will be contacted.

Electronic data generated by the Test Facility were archived as noted above.

8. RESULTS

For detailed results see Appendix 1.

8.1. Pilot Study

At 2000 mg/kg, no mortality occurred and no significant clinical signs were observed during the observation period.

8.2. Main Study

8.2.1. Mortality

No mortality occurred.

8.2.2. Clinical Observations

Hunched posture and piloerection were noted for the animals on Day 1.

8.2.3. Body Weights

The body weight gain shown by the animals over the study period was considered to be similar to that expected of normal untreated animals of the same age and strain.

8.2.4. Macroscopic Findings

No abnormalities were found at macroscopic post mortem examination of the animals.

9. CONCLUSION

The minimum oral lethal dose of in rats was established to exceed 2000 mg/kg body weight. Based on the absence of mortality rate at 2000 mg/kg body weight, it was concluded that the oral LD50 value of exceeds 2000 mg/kg body weight.

According to the OECD 423 test guideline, the LD50 cut-off values was considered to exceed 2000 mg/kg body weight.

Based on these results, does not have to be classified and has no obligatory labelling requirement for acute oral toxicity according to the:

- Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations (2017) (including all amendments),
- Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (including all amendments).

Appendix 1
Tables

TABLE 1 MORTALITY DATA

TEST DAY HOURS AFTER TREATMENT	1 0	1 2	1 4	2	3	4	5	6	7	8	9	10	11	12	13	14	15
FEMALES 2000 MG/KG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FEMALES 2000 MG/KG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 2 CLINICAL SIGNS

TEST DAY HOURS AFTER TREATMENT	MAX GRADE	1 0	1 2	1 4	2	3	4	5	6	7	8	9	10	11	12	13	14	15
FEMALES 2000 MG/KG																		
ANIMAL 1																		
Posture																		
Hunched posture	(1)	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skin / fur																		
Piloerection	(1)	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FEMALES 2000 MG/KG																		
ANIMAL 2																		
Posture																		
Hunched posture	(1)	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skin / fur																		
Piloerection	(1)	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ANIMAL 3																		
Posture																		
Hunched posture	(1)	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skin / fur																		
Piloerection	(1)	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ANIMAL 4																		
Posture																		
Hunched posture	(1)	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skin / fur																		
Piloerection	(1)	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ANIMAL 5																		
Posture																		
Hunched posture	(1)	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skin / fur																		
Piloerection	(1)	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = Sign not observed

TABLE 3 BODY WEIGHTS (GRAM)

SEX/DOSE LEVEL	ANIMAL	DAY 1	DAY 8	DAY 15
FEMALES 2000 MG/KG				
	1	160	199	212
	MEAN	160	199	212
	ST.DEV.	---	---	---
	N	1	1	1
FEMALES 2000 MG/KG				
	2	170	201	213
	3	200	236	244
	4	164	184	206
	5	176	200	231
	MEAN	178	205	224
	ST.DEV.	16	22	17
	N	4	4	4

TABLE 4 MACROSCOPIC FINDINGS

ANIMAL ORGAN	FINDING	DAY OF DEATH
FEMALES 2000 MG/KG		
1	No findings noted	Scheduled necropsy Day 15 after treatment
FEMALES 2000 MG/KG		
2	No findings noted	Scheduled necropsy Day 15 after treatment
3	No findings noted	Scheduled necropsy Day 15 after treatment
4	No findings noted	Scheduled necropsy Day 15 after treatment
5	No findings noted	Scheduled necropsy Day 15 after treatment

Appendix 2
Test Item Characterization

Appendix 3
Study Plan

FINAL STUDY PLAN

Test Facility Study No. 20146048

Sponsor Reference No.

An Acute Study of by Oral Gavage in Rat (Fixed Dose Method)

TEST FACILITY:
Charles River Laboratories Den Bosch B.V.
Hambakenwetering 7
5231 DD ‘s-Hertogenbosch
The Netherlands

TABLE OF CONTENTS

1. OBJECTIVE(S)

The objective of this study is to determine the potential toxicity of when given by oral gavage at a single dose to rats of a single sex at one or more defined doses to evaluate the potential reversibility of any findings. This study is intended to provide information on the potential health hazards of and data produced can be used for classification/labelling of the test item. This study should provide a rational basis for risk assessment in man.

2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual applicable dates will be included in the Final Report.

Experimental Start Date:	05 April 2018 (Week 14) (First date of study-specific data collection; weighing of test item)
Experimental Completion Date:	03 June 2018 (Week 22) (Last date data are collected from the study; necropsy)
Initiation of Dosing	09 Apr 2018 (Week 15)
Completion of In-life	27 May 2018 (Week 21) (Last date of necropsy)
Unaudited Draft Report:	10 Jun 2018 (Week 23)

3. GUIDELINES FOR STUDY DESIGN

The design of this study was based on the study objective(s), the overall product development strategy for the test item and in compliance with the following study design guidelines:

- OECD Guideline 420. *Acute Oral Toxicity – Fixed Dose Procedure*, December 2001.
- EPA Health Effects Test Guideline OPPTS 870.1100. *Acute Oral Toxicity in Rodents*, December 2002.
- EC No 440/2008 Part B. *Acute Oral Toxicity, Fixed Dose Procedure,* May 2008.
- Appendix to Director General Notification, No. 12-Nousan-8147. Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan (JMAFF), November 2000.

4. REGULATORY COMPLIANCE

The study will be performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America, Japan, and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

5. QUALITY ASSURANCE

5.1. Test Facility

The Test Facility Quality Assurance Unit (QAU) will monitor the study to assure the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the study plan, conduct study and/or process inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

The Test Facility QAU contact is indicated below:

C.J. Mitchell, BSc.
Address as cited for Test Facility
Tel: +31 73 640 6700
E-mail: Christine.Mitchell@crl.com

6. SPONSOR

Sponsor Representative / Sponsor Study Monitor

7. RESPONSIBLE PERSONNEL

Study Director

P.H.T. van Sas, MSc.
address as cited for Test Facility
Tel: +31 73 640 6700
E-mail: Pieter.vanSas@crl.com

Management Contact

H.H. Emmen, MSc.
address as cited for Test Facility
Tel: +31 73 640 6700
E-mail: Harry.Emmen@crl.com

8. TEST ITEM

8.1. Test Item

Identification:

Appearance:	Clear colourless liquid
Batch:	12639
Purity/Composition:	See Certificate of Analysis
Test item storage:	At room temperature container flushed with nitrogen
Stable under storage conditions until:	08 November 2020 (expiry date)
Additional information	
Test Facility test item number:	209150/A
Purity/Composition correction factor:	No correction factor required
Test item handling:	Handle in glove box (nitrogen environment)
Chemical name (IUPAC, synonym or trade name:	

8.2. Test Item Characterization

The Sponsor will provide to the Test Facility documentation of the identity, purity, composition, and stability for the test item. If available, a Certificate of Analysis or equivalent documentation will be provided for inclusion in the Final Report. The Sponsor will also provide information concerning the regulatory standard that was followed for these evaluations.

The Sponsor has appropriate documentation on file concerning the method of synthesis, fabrication or derivation of the test item, and this information is available to the appropriate regulatory agencies should it be requested.

8.3. Analysis of Test Item

The stability of the bulk test item will not be determined during the course of this study. Information to support the stability of each lot of the bulk test item will be provided by the Sponsor.

8.4. Reserve Samples

For each batch (lot) of test item, if practically possible a reserve sample will be collected and maintained under the appropriate storage conditions by the Test Facility and destroyed after the expiration date.

8.5. Test Item and Vehicle Inventory and Disposition

Records of the receipt, distribution, storage, and disposition of test item will be maintained.

9. SAFETY

The following safety instructions apply to this study:

Standard safety precautions specified in Charles River Den Bosch procedures. Specific safety precautions are provided in the Charles River Den Bosch internal EH&S test item risk assessment.

10. DOSE FORMULATION AND ANALYSIS

10.1. Preparation of Test Item

The test item, will be administered as received.

Adjustment will be made for specific gravity of the test item.

Any residual volumes will be discarded unless otherwise requested by the Study Director.

10.2. Sample Collection and Analysis

The test item will be used as received from the Sponsor; therefore, samples for dose formulation analysis will not be collected by the Test Facility.

11. TEST SYSTEM

Species:	Rat
Strain:	Crl: WI(Han)
Condition:	Outbred, SPF-Quality
Source:	Based on availability, one of the following sources will be used and specified in the report: • Charles River France, L'Arbresle, France • Charles River Deutschland, Sulzfeld, Germany
Number of Animals:	Pilot Study: 5 females (maximum) Main Study: 5 females per dose group. Including the single female that is dosed in the pilot study at the selected concentration. Females will be nulliparous and non-pregnant.
Target Age at the Initiation of Dosing:	Between 8 and 12 weeks old. Animals to be used within the study will be of approximately the same age.
Target Weight at the Initiation of Dosing:	150 to 300 g.

The actual age and weight of animals dosed will be listed in the Final Report.

11.1. Justification of Test System and Number of Animals

The Wistar Han rat was chosen as the animal model for this study as recognized by international guidelines as a recommended test system. The test method and number of animals are based on the test guidelines.

11.2. Animal Identification

At study assignment, each animal will be identified using an ear mark and tail mark with indelible ink.

11.3. Environmental Acclimation

The animals will be allowed to acclimate to the Test Facility toxicology accommodation for at least 5 days before the commencement of dosing.

11.4. Selection, Assignment, Replacement, and Disposition of Animals

Animals will be assigned to the study at the discretion of the coordinating biotechnician according to body weights, with all animals within ± 20% of the sex mean. Animals in poor health or at extremes of body weight range will not be assigned to the study.

Before the initiation of dosing, a health inspection will be performed and any assigned animals considered unsuitable for use in the study will be replaced by alternate animals obtained from the same shipment and maintained under the same environmental conditions.

The disposition of all animals will be documented in the study records.

12. HUSBANDRY

12.1. Housing

On arrival, animals will be group housed (up to 5 animals of the same sex together) in polycarbonate cages (Makrolon MIV type; height 18 cm.) and following assignment to the study, animals will be individually housed (pilot study) or group housed (main study, up to 5 animals of the same sex and same dosing group together) in polycarbonate cages (Makrolon MIV type; height 18 cm.) containing sterilized sawdust as bedding material (Lignocel S 8-15, JRS - J.Rettenmaier & Söhne GmbH + CO. KG, Rosenberg, Germany) equipped with water bottles. These housing conditions will be maintained unless deemed inappropriate by the Study Director and/or Clinical Veterinarian. The room(s) in which the animals will be kept will be documented in the study records.

Animals will be separated during designated procedures/activities. Each cage will be clearly labeled.

12.2. Environmental Conditions

The target conditions for animal room environment will be as follows:

Temperature:	18 to 24°C
Humidity:	40 to 70%
Light Cycle:	12-hours light and 12-hours dark (except during designated procedures)
Ventilation:	At least 10 air changes per hour

12.3. Food

Pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany) will be provided ad libitum throughout the study, except during designated procedures.

The feed is analyzed by the supplier for nutritional components and environmental contaminants. Results of the analysis are provided by the supplier and are on file at the Test Facility.

It is considered that there are no known contaminants in the feed that would interfere with the objectives of the study.

12.4. Water

Municipal tap-water will be freely available to each animal via water bottles.

Periodic analysis of the water is performed, and results of these analyses are on file at the Test Facility.

It is considered that there are no known contaminants in the water that would interfere with the objectives of the study.

12.5. Animal Enrichment

For psychological/environmental enrichment, animals will be provided with paper (Enviro-dri, Wm. Lillico & Son (Wonham Mill Ltd), Surrey, United Kingdom), except when interrupted by study procedures/activities.

12.6. Veterinary Care

Veterinary care will be available throughout the course of the study and animals will be examined by the veterinary staff as warranted by clinical signs or other changes. All veterinary examinations and recommended therapeutic treatments, if any, will be documented in the study records.

13. EXPERIMENTAL DESIGN

In principle the study comprises two stages: a pilot Study and a Main Study.

In order to set the dose level for the main study, a pilot study will be performed in single animals. The following fixed dose levels are considered: 5, 50, 300 and 2000 mg/kg. Administration will normally be started at 2000 mg/kg body weight in one female rat. If death or severe effects necessitating humane killing are seen, then single animals will be treated with sequentially lower fixed dose levels.

The main study will be conducted at the highest dose level, which produces no mortality.

The methods used in the pilot study will be similar to those used in the main study, except for individual housing of the pilot animal(s). A summary of the results will be reported.

13.1. Administration of Test item

A single dose of test item will be administered to the appropriate animals by oral gavage on Day 1, using a syringe with a plastic gavage cannula attached. The Starting dose level will be 2000 mg/kg body weight.

The dose volume for each animal will be based on the body weight measurement prior to dosing. Dose volume (mL/kg body weight) will be calculated as follows:
Dose level (g/kg) / spec.gravity or density (g/mL)

The dosing formulations will be stirred continuously during dose administration.

13.2. Justification of Route and Dose Levels

The oral route is selected as it is a possible route of human exposure during manufacture, handling or use of the test item.

The dose level will be based on the results of the pilot study and will be specified and approved by the Study Director in the study files. If at the selected dose level no evident toxicity is seen or mortality occurs, a next higher or lower dose level may be tested, respectively. The highest dose level will be 2000 mg/kg body weight.

14. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS

14.1. Mortality/Moribundity Checks

Frequency:	Twice daily throughout the study.
Procedure:	Animals will be observed for general health/mortality and moribundity. Animals will not be removed from cage during observation, unless necessary for identification or confirmation of possible findings.

14.2. Clinical Observations

14.2.1. Postdose Observations

Frequency:	At periodic intervals on the day of dosing (at least three times) and at least once daily thereafter. The observation period will be 14 days.
Procedure:	All the animals will be examined for reaction to dosing. The onset, intensity and duration of these signs will be recorded (if appropriate), particular attention being paid to the animals during and for the first hour after dosing.

14.3. Body Weights

Frequency:	On Days 1 (predose), 8 and 15.
Procedure:	Animals will be individually weighed. A fasted weight will be recorded on the day of dosing. Terminal body weights will also be collected from animals if found dead or euthanized moribund after Day 1.

15. TERMINAL PROCEDURES

All moribund animals and animals surviving to the end of the observation period will be sacrificed by oxygen/carbon dioxide procedure. All animals assigned to the study are subjected to necropsy and descriptions of all internal macroscopic abnormalities will be recorded.

16. ANALYSIS

The results can be evaluated according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations (including all amendments) and the Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of items and mixtures (including all amendments).

17. COMPUTERIZED SYSTEMS

The following critical computerized systems may be used in the study. The actual critical computerized systems used will be specified in the Final Report.

Data for parameters not required by study plan, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by study plan and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

Critical Computerized Systems

System Name	Description of Data Collected and/or Analyzed
REES Centron	Temperature and Humidity (Animal and Laboratory facilities) Data Collection
TOXDATA[1]	In-life phase (mortality; clinical signs; body weights) data collection

1. For logistic reasons, data will be captured under study number 520768. All data will be reported under Test Facility Study No. 20146048.

18. AMENDMENTS AND DEVIATIONS

Changes to the approved study plan shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary study plan changes in advance with the Sponsor.

All study plan and SOP deviations will be documented in the study records. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

19. RETENTION OF RECORDS

All study-specific raw data, electronic data, documentation, study plan and final reports will be archived by no later than the date of Final Report issue. All materials generated by Charles River from this study will be transferred to a Charles River archive. At least five years after issue of the Final Report, the Sponsor will be contacted.

Records to be maintained will include, but will not be limited to, documentation and data for the following:

- Study plan, study plan amendments, and deviations
- Study schedule
- Study-related correspondence
- Test system, health, and husbandry
- Test item receipt, identification, preparation
- In-life measurements and observations
- Gross observations and related data

20. REPORTING

A comprehensive Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft Report. The Final Report will be provided in Adobe Acrobat PDF format (hyperlinked and searchable) along with a Microsoft Word version of the text. The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Test Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation. An original copy of the report with the Test Facility's handwritten signatures will be retained.

Reports should be finalized within 6 months of issue of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Test Facility unless other arrangements are made by the Sponsor.

21. ANIMAL WELFARE

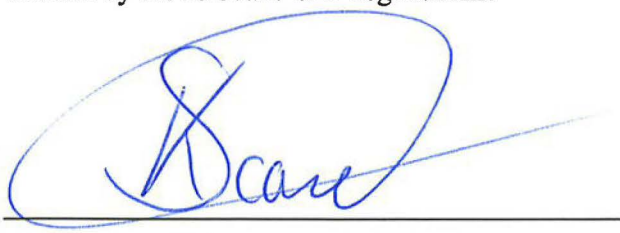
This study plan was reviewed and agreed by the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. within the framework of project license AVD2360020172866 (Appendix 1) approved by the Central Authority for Scientific Procedures on Animals (CCD) as required by the Dutch Act on Animal Experimentation (December 2014).

Animals showing pain, distress or discomfort, which is considered not transient in nature or is likely to become more severe, will be sacrificed for humane reasons based on OECD guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation (ENV/JM/MONO/ 2000/7).

By approving this study plan, the Sponsor affirms that this study is required by a relevant government regulatory agency and that it does not unnecessarily duplicate any previous experiments.

TEST FACILITY APPROVAL

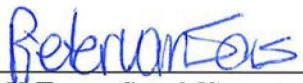
The signature below acknowledges Test Facility Management's responsibility to the study as defined by the relevant GLP regulations.



Date: 04-Apr-2018

K. Scase, MSc.
Section Head General Toxicology

The signature below indicates that the Study Director approves the study plan.



Date: 04 Apr 2018

P.H.T. van Sas, MSc.
Study Director

ATTACHMENT A

Distribution List

Electronic copies will be supplied unless otherwise specified below.

Version	**Recipient**	
Original	Study Director	
1 Copy	Sponsor Representative / Study Monitor	
1 Copy	QAU / Management	
1 Copy	Necropsy	HER/necropsy;
1 Copy	Formulations	Tsfher;
1 Paper Copy	Coordinating Biotechnician	van Voorden, K;

TRADE SECRET

Study Title

CAS# A 72-HOUR TOXICITY TEST WITH THE FRESHWATER ALGA (*Raphidocelis subcapitata*)

Test Guidelines

OECD Guideline 201 (2011)

Authors

Joshua R. Arnie B.S. (Study Director)
Laura A. Lockard, M.S.
John A. Aufderheide, B.A.
Kathy H. Martin, M.S.

Study Completion Date

October 19, 2018

Test Facility

EAG, Inc.
8598 Commerce Drive
Easton, Maryland 21601
U.S.A.

Sponsor

Performing Laboratory Study Number

783P-102

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The study described in this final report was conducted in compliance with U.S. EPA Good Laboratory Practice Standards (40 CFR part 792); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17), and MAFF (11 Nousan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999).

Periodic analyses of the water for potential contaminants were not performed according to Good Laboratory Practice Standards, but were performed using a certified laboratory and standard US EPA analytical methods.

Study Director

Joshua R. Arnie, B.S.
Manager of Plant Toxicology
EAG Laboratories – Easton

19 OCTOBER 2018
Date

QUALITY ASSURANCE STATEMENT

Study Number

EAG Laboratories – Easton Project No.: 783P-102

The Study No.: 783P-102, entitled "CAS #39654-39-2: A 72-Hour Toxicity Test with the Freshwater Alga (*Raphidocelis subcapitata*)" has been inspected in accordance with the OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17), the U.S. Environmental Protection Agency (40 CFR Part 792, 17 August 1989); and Japan MAFF (11 Nousan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999).

This study was inspected and findings reported to Management and to the Study Director on the dates shown below:

Activity	*Audit Dates**	*Dates Findings Reported to Study Director*	*Dates Findings Reported to Management*
Protocol	February 21, 2018	February 21, 2018	April 06, 2018
Test Solution Preparation	June 19, 2018	June 19, 2018	June 21, 2018
pH Measurements	June 22, 2018	June 22, 2018	July 02, 2018
Analytical Data & Draft Report	September 06 and 07, 2018	September 14, 2018	September 07, 2018
Biological Data & Draft Report	October 4-5, 2018	October 5, 2018	October 10, 2018
Final Report	October 19, 2018	October 19, 2018	October 19, 2018

* All inspections were study-based unless otherwise noted.

These inspections confirm that the methods, procedures, and observations are accurately and completely described in this final report, and the reported results accurately and completely reflect the raw data of this study.

for

Nicholas R. Gardner
Quality Assurance Representative I
EAG Laboratories – Easton

October 19, 2018
Date

CERTIFICATION OF AUTHENTICITY

CAS# 39654-39-2: A 72-HOUR TOXICITY TEST WITH THE FRESHWATER ALGA (*Raphidocelis subcapitata*)

We, the undersigned, declare that the work described in this final report was performed under our supervision, and that this final report provides an accurate record of the procedures and results.

Report by:

Joshua R. Arnie, B.S. (Study Director) — Date: 19 OCTOBER 2018
Manager of Plant Toxicology
EAG Laboratories – Easton

Laura A. Lockard, M.S. — Date: October 19, 2018
Staff Scientist II
EAG Laboratories-Easton

Approved by:

John A. Aufderheide, B.A. — Date: 19 OCTOBER 2018
Senior Director
EAG Laboratories – Easton

Kathy H. Martin, M.S. — Date: October 19, 2018
Associate Director of Chemistry
EAG Laboratories-Easton

Study Initiated:
June 18, 2018

Date Study Completed:
October 19, 2018

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BASIC STUDY INFORMATION

Study Title
CAS# A 72-Hour Toxicity Test with the Freshwater Alga (*Raphidocelis subcapitata*)

Study Objective
The objective of this study was to determine the toxicity of the test material, CAS# on the freshwater alga, (*Raphidocelis subcapitata*), during a 72-hour exposure period.

Study Director
Joshua R. Arnie, B.S.
EAG, Inc.
8598 Commerce Drive
Easton, Maryland 21601
U.S.A.

Laboratory Management, EAG Laboratories – Easton
John A. Aufderheide, B.A., Senior Director
Kathy H. Martin, M.S., Associate Director of Chemistry

Test Item
CAS#

Testing Facilities
EAG, Inc.
8598 Commerce Drive
Easton, Maryland 21601
U.S.A.

CAS# A 72-HOUR TOXICITY TEST WITH THE FRESHWATER ALGA (*Raphidocelis subcapitata*)

1.0 SUMMARY

SPONSOR:

STUDY: CAS# A 72-Hour Toxicity Test with the Freshwater Alga (*Raphidocelis subcapitata*)

EAG LABORATORIES PROJECT NUMBER: 783P-102

TEST DATES: Experimental Start (OECD) – June 19, 2018
Experimental Start (EPA) – June 19, 2018
Exposure Termination – June 22, 2018
Experimental Termination – June 22, 2018

LENGTH OF EXPOSURE: 72 Hours

TEST ORGANISMS: Freshwater algae (*Raphidocelis subcapitata*)

SOURCE OF TEST ANIMALS: EAG Laboratories
8598 Commerce Drive
Easton, MD 21601
U.S.A.

TEST CONCENTRATIONS:

Nominal (mg a.i./L)	Mean Measured (mg a.i./L)
Blank control (0.0)	<LOQ*
6.3	<LOQ*
13	<LOQ*
25	<LOQ*
50	<LOQ*
100	<LOQ*

*LOQ = limit of quantitation; The LOQ was determined to be 3.0 mg a.i./L.

RESULTS: Cell density increased in the blank control by a factor of 105 in 72 hours. The coefficient of variation of average specific growth rates during the whole test period (0-72 hour) in replicates of the blank control was 2.8%. In addition, the mean percent coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, and 2-3) was 52.4%, which exceeded the OECD 201 requirement of 35%. This study employed a closed-bottle test design where replicates were sampled every 24 hours and after sampling they were removed from the study. This particular criterion should not be considered applicable following this test design since it's impossible to monitor growth in the control replicates over time.

	Nominal Test Concentration
Yield:	72-hr E_yC_{50} > 100 mg a.i./L
	(n/a)
	72-hr NOEC = 100 mg a.i./L
Growth Rate:	72-hr E_rC_{50} > 100 mg a.i./L
	(n/a)
	72-hr NOEC = 100 mg a.i./L
Cell Density:	72-hr EC_{50} > 100 mg a.i./L
	(n/a)
	72-hr NOEC = 100 mg a.i./L

2.0 INTRODUCTION

This study was conducted by EAG Laboratories for at the EAG Laboratories aquatic toxicology facility in Easton, Maryland. The in-life phase of the definitive test was conducted from June 19 to 22, 2018, with cell counts completed on June 22, 2018. The raw data generated at EAG Laboratories and a copy of the final report are filed under Project Number 783P-102 in the archives located at the EAG Laboratories-Easton site.

3.0 OBJECTIVE

The objective of this study was to determine the toxicity of CAS# to the freshwater alga, *Raphidocelis subcapitata*, over a 72-hour exposure period.

4.0 EXPERIMENTAL DESIGN

The green alga, *Raphidocelis subcapitata*, was exposed to five test concentrations and a blank control (culture medium) under static conditions for 72 hours. Six replicate test chambers in the control group and three replicate test chambers in each treatment (test concentration) group were included for each 24-hour sampling interval. CAS# is volatile, therefore, the study was conducted in a closed system in an attempt to maintain exposure concentrations. There were a total of nine replicates in each treatment group and 18 blank control replicates at test initiation. Only 17 blank control replicates were inadvertently prepared at test initiation due to biologist oversight. This deviation from the approved protocol was not considered to be detrimental to the study. Nominal test concentrations were selected in consultation with the Sponsor and were based upon the results of an exploratory range-finding toxicity test (see results below).

Nominal Concentration (mg/L)	72-Hour Cell Density (cells/mL)	Percent Inhibition vs. Control
Blank control	715,000	--
1.0	640,000	10
10	670,000	-5
100	500,000	25

At test initiation an inoculum of the algal cells was added to each test chamber to achieve a nominal concentration of approximately 5,000 *Raphidocelis* cells/mL. Samples were collected from the appropriate replicate test chambers (6 control, 3 per treatment) at approximately 24-hour intervals during the test to determine cell densities. Cell densities were used to determine growth rates and yields which were subsequently used to calculate percent inhibition values relative to the blank control over the 72-hour exposure period. EC_{50} and E_rC_{50} values (i.e., the theoretical concentrations that would produce an 50% reduction in cell density and growth rate, respectively) and their 95% confidence intervals were determined, when possible, at 24, 48, and 72 hours of exposure. The 72-hour E_yC_{50} value and corresponding 95% confidence interval, (i.e., the theoretical concentrations that would produce a 50% reduction in cell yield) were determined. No-observed-effect-concentrations (NOEC) were determined at 72 hours through statistical evaluation of the cell density, growth rate, and yield data, as well as examination of the concentration-response pattern.

5.0 MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "CAS# A 72-Hour Toxicity Test with the Freshwater Alga (*Raphidocelis subcapitata*)". The protocol was based on procedures outlined in OECD Guidelines for Testing of Chemicals, 201: *Freshwater Alga and Cyanobacteria, Growth Inhibition Test* (1) and the Official Journal of the European Communities No. L 383 A, Method C.3.: *Algal Inhibition Test* (2). Changes to the study protocol are presented in Appendix 4.

5.1 Test Substance

The test substance used to prepare the test solutions, the analytical matrix fortification samples and the analytical calibration standards for the study was received from on April 6, 2018. It was assigned EAG Laboratories identification number 14633 upon receipt and was stored under ambient conditions. The test substance, a liquid, was identified as: CAS # Lot number Sample# 12639. The test substance contained 99.09% active ingredient (Appendix 2).

5.2 Test Organism

The freshwater alga, *Raphidocelis subcapitata*, was selected as the test species for this study. The species is representative of an important group of freshwater algae,

and was selected for use in the test based upon a past history of use, and ease of culturing in the laboratory. Original algal cultures were obtained from the University of Texas at Austin, and have been maintained in culture medium at EAG Laboratories, Easton, Maryland since June 2017. Algal cells used in this test were obtained from EAG Laboratories – Easton cultures that had been actively growing in culture medium under similar environmental conditions as used in this test for at least two weeks prior to test initiation. Algal cells for this study were taken from a culture that had been transferred to fresh medium four days prior to test initiation.

5.3 Freshwater (AAP) Medium Preparation

The algal cells were cultured and tested in freshwater AAP medium (1). Stock nutrient solutions were prepared by adding reagent-grade chemicals to purified EAG Laboratories-Easton well water (NANOpure® water). The test medium then was prepared by adding appropriate volumes of the stock nutrient solutions to NANOpure® water (Appendix 2). Additional sodium bicarbonate was added to the medium (35 mg/L; 50 mg/L total) to facilitate the closed-bottle test design. The pH of the medium was adjusted to 7.5 with 10% hydrochloric acid. The medium was sterilized by filtration (0.22 µm) and stored refrigerated prior to use. The most recent analyses performed to measure the concentrations of selected contaminants in the well water used to prepare the nutrient medium are presented in Appendix 3.

5.4 Test Apparatus

Test chambers were sterile, 300-mL glass bottles sealed with glass stoppers, and were completely filled, leaving minimal headspace. The test chambers contained 2 glass marbles to promote mixing. The test chambers were labeled with the project number, test concentration, and replicate, and were indiscriminately positioned daily in an environmental chamber designed to maintain the desired test temperature throughout the test. The test flasks were continuously shaken on a mechanical shaker at 100 rpm throughout the duration of the test.

5.5 Preparation of Test Concentrations

Test solutions were prepared by direct addition of calculated volumes of CAS# with Hamilton gas-tight syringes to freshwater AAP medium with additional sodium bicarbonate in 4 L glass aspirator bottles which were filled leaving no headspace. The target nominal concentrations were 6.3, 13, 25, 50, and 100 mg a.i./L. The calculated amounts of test substance were determined based on the reported density of the test substance (1.6194 g/cm^3) and the reported test substance purity (99.09%). The test solutions stirred with a Teflon® lined stir bar on a magnetic stir plate for approximately 15 minutes. The test solutions appeared clear and colorless with no precipitates. The negative control solution contained freshwater AAP medium with additional sodium bicarbonate only.

5.6 Analytical Sampling

Samples of the test solutions were collected to measure concentrations of the test substance at approximately 0, 24, 48, and 72 hours of exposure. Duplicate samples were collected at each sampling interval with one set of samples processed immediately for analysis and the other set stored refrigerated for potential future

analysis if deemed necessary by the Study Director. Samples collected at test initiation (0 hour) were collected from the individual batches of test solution prepared for each treatment and control group prior to distribution into the test chambers. Samples collected at 24, 48, and 72 hours were collected from the pooled replicates from each treatment and control group that were sampled for cell density determinations at that exposure interval. At each sampling interval, test solution samples were added to 20 mL glass scintillation vials containing toluene.

5.7 Analytical Method

Freshwater AAP medium samples of CAS# were analyzed by gas chromatography with electron capture detection (GC/ECD). A method outline is provided in Appendix 5, Figure 1.

Typical GC/MS Instrument and Conditions

INSTRUMENT:	Agilent model 7890A gas chromatograph (GC)
DETECTOR:	Electron Capture Detector (ECD)
ANALYTICAL COLUMN:	Agilent GS-Q analytical column (30 m x 0.32 µm)
INJECTOR TEMPERATURE:	220°C
RUN TIME:	11.2 minutes
OVEN:	Initial temperature: 45°C Initial time: 1.00 minute Ramp: 25°C/minute Final temperature: 250°C Final hold time: 2.00 minute
DETECTOR TEMPERATURE:	250°C
INJECTION VOLUME:	2 µL
CARRIER GAS:	Helium, 12 psi
MAKEUP GAS:	Nitrogen
APPROXIMATE RETENTION TIME:	7.8 minutes

Calibration standards of CAS# ranging in concentration from 1.00 to 50.0 mg a.i./L, were prepared in toluene using a stock solution of CAS# in acetone. A calibration curve was constructed for each set of analysis. The peak area and the theoretical concentrations of the calibration standards were fit with least-squares regression analysis to a weighted (1/x) linear function. The concentrations of CAS# in the samples were determined by substituting the peak area responses of the samples into the applicable linear regression equations. Samples were diluted, as necessary, into the calibration standard range using dilution factors ranging from 1.00 to 3.00.

The method limit of quantitation (LOQ) for CAS# in freshwater AAP medium was set at 3.00 mg a.i./L, based upon the lowest analyte concentration in a fortified sample that obtained a mean recovery of 70-110%. Matrix blank samples were analyzed to determine possible interferences.

The signal-to-noise (S/N) ratios for four injections of the lowest calibration standard were determined. The limit of detection (LOD) was calculated for each standard by dividing the standard concentration by the S/N ratio x 3 x the dilution factor (1.00) of the matrix blank samples. The mean LOD for CAS# was calculated and reported as 0.0664 mg a.i./L.

Samples were fortified at 3.00 and 100 mg a.i./L using stock solutions of CAS# in acetone and were analyzed for CAS# with the sample sets.

Example Calculations

An example of a typical calculation used in the analysis of CAS# for sample number 783P-102-3 with a nominal concentration of 13 mg a.i./L follows:

Concentration of CAS# in sample (mg a.i./L) =

$$\frac{\text{peak area - (y-intercept)}}{\text{slope}} \text{ x dilution factor}$$

Percent of nominal concentration =

$$\frac{\text{Measured concentration of sample (mg a.i./L)}}{\text{Nominal concentration of sample (mg a.i./L)}} \text{ x } 100$$

Peak Area = 778.92456
Y-Intercept = 256
Slope = 6514
Dilution Factor = 1.00

Concentration of CAS# in sample (mg a.i./L) = $\frac{778.92456 - 256}{6514}$ x 1.00

Concentration of CAS# in sample (mg a.i./L) = 0.0802* (< LOQ)

CAS# concentration = $\frac{0.0802 \text{ mg a.i./L}}{13 \text{ mg a.i./L}}$ x 100

Percent of nominal CAS# concentration = 0.617%*

* Results were generated using Excel 2010 in full precision mode. Manual calculations may differ slightly.

Analytical Stocks and Standards Preparation

A stock solution of CAS# was prepared by accurately weighing 1.0093 g of the test substance (weight corrected for 99.09% purity of the test substance) on an analytical balance. The test substance was transferred to a 100 mL volumetric flask and the contents were brought to volume using acetone. The primary stock solution (10.0 mg a.i./mL) was diluted in acetone to prepare a 1.00 mg a.i./mL stock solution. The 1.00 and 10.0 mg a.i./mL stock solutions were used to fortify the quality control samples. The 1.00 mg a.i./mL stock solution was used to prepare the calibration standards in toluene. The following shows the dilution scheme for a set of calibration standards:

Stock Concentration (mg a.i./mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg a.i./L)
1.00	25.0	25.0	1.00
1.00	50.0	10.0	5.00
1.00	100	10.0	10.0
1.00	250	10.0	25.0
1.00	500	10.0	50.0

5.8 Inoculation of Test Chambers

Prior to test initiation, the concentration of algal cells in the composite culture of two stock cultures (culture identification No. 17-01 and 17-02) was determined using a hemacytometer and microscope, and was 9.3×10^5 cells/mL. In order to achieve the desired initial cell density of approximately 5,000 cells/mL, 1.6 mL of stock culture was added to each replicate test chamber at test initiation with an Eppendorf pipette.

5.9 Environmental Conditions

Test chambers were held in an environmental chamber at a temperature of 24 ± 2ºC. The temperature of a container of water adjacent to the test chambers in the environmental chamber was measured continuously using an Amega Scientific Corporation centralized monitoring system. The algae were held under continuous cool-white fluorescent lighting throughout the test. The target light intensity was 6,000 lux ± 10%. Light intensity was measured at test solution level at five locations surrounding the test flasks at test initiation using a SPER Scientific 840006C light meter. The pH of the medium in each treatment and control group was measured at test initiation and exposure termination using a Thermo Orion A214 pH meter. At test initiation, pH was measured in the individual batches of test solution prepared for each treatment and control group. At exposure termination, pH was measured in pooled samples of test solution collected from the remaining replicates included in each treatment and control group.

5.10 Algal Growth Measurements and Observations

Test samples were collected from designated replicates of the test concentrations and control for the determination of algal cell densities. Samples were collected at

approximately 24-hour intervals during the 72-hour exposure. Cell samples were stored under refrigerated conditions and in the dark after collection. Samples collected for the enumeration of cells were stored for a maximum of two days until cell counts were performed. Prior to counting, the sample solutions were sonicated for approximately one minute and shaken to mix. Cell counts for the samples collected during the exposure phase were performed using an electronic particle counter (Coulter Electronics, Inc.). Prior to conducting cell counts, the linearity of the instrument response was determined at settings previously established for *R. subcapitata*. A primary counting standard containing *R. subcapitata* cells was prepared, the density was verified using a hemocytometer and a microscope, and the standard was subsequently diluted to provide a series of seven counting standards for the determination of instrument linearity. Theoretical densities were assigned to each secondary counting standard based upon the verified density of the primary counting standard and the dilution ratio. The cell densities of the counting standards were measured using the electronic particle counter and were compared to the theoretical densities by performing a least squares regression analysis. Cell counts for samples collected during the test were conducted once instrument linearity was demonstrated (i.e., the R-squared value obtained through the regression analysis was 0.99951). A single aliquot of each sample collected during the test was diluted with an electrolyte solution (Isoton II®). Three 0.5-mL volumes of the diluted sample were counted, and the resulting counts were averaged. The cell density of the sample was determined by adjusting the mean cell count (cells/mL) obtained using the particle counter, based upon the y-intercept and slope calculated through the regression analysis, and the dilution factor. The following equation was used:

$$\text{Cell Density (cells/mL)} = \frac{\text{Mean Cell Count - Y Intercept}}{\text{Slope}} \text{ X Dilution Factor}$$

Microscopic examination of the inoculum culture was conducted prior to initiation of the test to assess whether the algae were normal in appearance. Samples of test solution were collected from the remaining biological replicates per treatment and control group at the end of the exposure period. These samples were pooled within their respective treatments, and subsamples were removed and examined microscopically for atypical cell morphology (e.g., changes in cell shape, size or color). Cells in the replicate test chambers also were assessed for aggregation, flocculation, or adherence of the cells to the test chamber.

5.11 Statistical Analyses

The calculation of cell densities, growth rates, yields and percent inhibition values, as well as all statistical analyses, were conducted using SAS Version 9.4 (3).

Yield was calculated for each treatment and control replicate at 72 hours as the final cell density in the respective exposure period minus the initial cell density.

Growth rates were calculated for each replicate of the control and test concentrations at 0-24, 0-48, and 0-72 hours using the following formula:

$$\mu = \frac{\ln N_n - \ln N_o}{t_n - t_o}$$

where:

μ	=	Average specific growth rate
N_o	=	Nominal cell density (cells/mL) at t_o
N_n	=	Measured cell density (cells/mL) at t_n
t_o	=	Time of beginning of test (hours)
t_n	=	Time after beginning of test (hours)

Inhibition values were calculated for each test concentration as the percent reduction in cell density, growth rate or yield relative to the blank control replicates using the following formula:

$$\text{Percent Inhibition} = \frac{\text{Mean Control Response - Mean Treatment Response}}{\text{Mean Control Response}} \text{ X } 100$$

EC_{50} values in this study represent the theoretical test concentrations that would produce a 50% reduction in a variable of interest relative to the blank control. EC_{50}, E_rC_{50}, and E_yC_{50} values and their corresponding 95% confidence intervals were calculated, when possible, using non-linear regression (4) with replicate data (cell density, growth rate, and yield, respectively) and nominal test concentrations.

The 72-hour cell density, growth rate, and yield data were evaluated for normality and homogeneity of variance ($\alpha = 0.01$) using Shapiro-Wilk's and Levene's tests, respectively. All data met assumptions of normality and homogeneity of variance. The mean treatment group responses were compared to the blank control response using Dunnett's one-tailed t-test ($\alpha = 0.05$). The results of the statistical analyses of the cell density, growth rate, and yield data, as well as an evaluation of the concentration-response pattern, were used to determine the NOEC relative to each parameter at 72 hours.

6.0 RESULTS AND DISCUSSION

6.1 Analytical Results

Chromatographic Results

CAS# eluted as a well-resolved peak with a retention time of approximately 7.8 minutes. The LOQ was 3.00 mg a.i./L.

Test Solution Results

The recoveries of CAS# in the test samples were below the limit of quantitation (Table 1).

The measured concentration for the matrix fortification samples analyzed for CAS# ranged from 73.6 to 103% of nominal concentrations (Table 2).

Blank control solutions showed no detectable levels of CAS# (Table 2).

A representative calibration curve for CAS# is presented in Appendix 5, Figure 2. Representative chromatograms of low and high-level calibration standards are presented in Appendix 5, Figures 3 and 4, respectively. Representative chromatograms of a matrix blank sample and a matrix fortification sample are presented in Appendix 5, Figures 5 and 6, respectively. A representative chromatogram of a test sample analyzed for CAS# is presented in Appendix 5, Figure 7.

6.2 Biological Results

At test initiation all test solutions appeared clear and colorless and otherwise unremarkable. At test termination, no particulates or surface slicks were observed in any of the treatment groups.

Measurements of temperature, pH, and light intensity are presented in Tables 3, 4, and 5, respectively. Water temperatures in a container of water located adjacent to the test ranged from 24.40 to 24.55°C over the 72-hour exposure period and were within the 24± 2°C range established for the test. The pH of test solutions at test initiation ranged from 7.6 to 7.8. At exposure termination, the pH of pooled remaining replicates of each experimental group ranged from 10.0 to 10.3. The observed increase in pH over the course of the test in each test substance concentration is typical for tests conducted with *R. subcapitata* and is attributed to the photosynthetic activity of the algae. The light intensity ranged from 5,440 to 6,420 lux, which was within the desired range of 6,000 ± 600 lux.

The toxicity of CAS# to *R. subcapitata* was determined by evaluating changes in cell density over a 72-hour exposure period. Cell densities were used to calculate growth rates for each 24-hour interval of exposure and yields at 72 hours of exposure. Mean cell densities and yields with the corresponding inhibition values, are presented in Table 6. Mean growth rates and corresponding percent inhibition values are presented in Table 7 EC_{50}, E_yC_{50} and E_rC_{50} values and the corresponding 95% confidence limits for cell density, yield and growth rates, respectively, as well as NOEC values at 72-hours are presented in Table 8. Individual replicate data for cell density and yield are presented in Appendix 6, while individual replicate data for growth rate is presented Appendix 7. Mean cell densities are illustrated graphically in Figure 1 for the blank control, and in Figure 2 for the blank control and each test concentration. Changes in mean cell density in the blank control replicates indicated that exponential growth of cells occurred in those replicates over the 72-hour exposure (Figure 1). Section-by-section specific growth rates for each replicate of the blank control group and the coefficient of variation of average specific growth rates for the blank control replicates during the whole test period are presented in Appendix 8.

After 72 hours of exposure, inhibition of cell density in the 6.3, 13, 25, 50, and 100 mg a.i./L treatment groups was -43, -30, -55, -25, and -29%, respectively, relative to the blank control (Table 6). Inhibition of yield in the 6.3, 13, 25, 50, and 100 mg a.i./L treatment groups was -43, -31, -55, -25, and -30%, respectively, relative to the blank control (Table 6). Inhibition of growth rate in the 6.3, 13, 25, 50, and 100 mg a.i./L treatment groups was -8, -6, -10, -5, and -5%, respectively, relative

to the blank control (Table 7). Mean cell density, mean yield, and mean growth rate were not significantly reduced (Dunnett's one-tailed t-test; $p < 0.05$) in any of the CAS# treatment groups at 72 hours, when compared to the blank control. Consequently, the 72-hour NOEC was determined to be 100 mg a.i./L.

At test initiation, algal cells in the inoculum appeared normal. On the day of test termination, microscopic observations were made from pooled samples of test solution collected from each treatment and control group. At test termination, cells in the CAS# treatment groups were observed to be normal in size, shape, and color when compared to cells present in the blank control. There was no evidence of aggregation of cells in any test group. Adherence of cells to the test vessels was observed in all experimental groups included the blank control, therefore, this was not considered to be a treatment related effect.

6.3 Conditions for the Validity of the Test

The test met the following criteria used to judge the validity of the test:
Cell density increased in the blank control by a factor of 105 in 72 hours. The coefficient of variation of average specific growth rates during the whole test period (0-72 hour) in replicates of the blank control was 2.8%. In addition, the mean percent coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, and 2-3) was 52.4%, which exceeded the OECD 201 requirement of 35%. This study employed a closed-bottle test design where replicates were sampled every 24 hours and after sampling they were removed from the study. This particular criterion should not be considered applicable following this test design since it's impossible to monitor growth in the control replicates over time.

7.0 CONCLUSIONS

The freshwater alga, *Raphidocelis subcapitata*, was exposed to a geometric series of five nominal measured concentrations of CAS# ranging from 6.3 to 100 mg a.i./L. Effects were evaluated based on cell density, yield, and growth rate using nominal test concentrations. The 72-hour EC_{50}, E_yC_{50}, and E_rC_{50} values were all determined to be >100 mg a.i./L. The 72-hour NOEC was determined to be 100 mg a.i./L.

8.0 REFERENCES

1. **Organisation for Economic Cooperation and Development**. 2011. OECD Guidelines for Testing of Chemicals, 201: *Freshwater Alga and Cyanobacteria, Growth Inhibition Test*. Adopted 23 March 2006. Revised 28 July 2011.
2. **Official Journal of the European Communities.** 1992. No. L 383 A. Method C.3. *Algal Inhibition Test.*
3. **SAS Institute, Inc.** 2002 - 2012. SAS System for Windows, Version 9.4. Cary, NC, SAS Institute, Inc.
4. **Bruce, Robert D. and Donald J. Versteeg.** 1992. A Statistical Procedure for Modeling Continuous Toxicity Data. *Environmental Toxicology and Chemistry.* 11: 1485-1494.

TABLE 1 MEASURED CONCENTRATIONS OF CAS# IN FRESHWATER AAP MEDIUM

Nominal CAS# Concentration (mg a.i./L)	Measured CAS# Concentration[1] (mg a.i./L)			
	Day 0 (% of Nominal)	Day 1 (% of Nominal)	Day 2 (% of Nominal)	Day 3 (% of Nominal)
Negative Control	ND[2]	ND[2]	ND[2]	ND[2]
6.3	ND[2]	ND[2]	ND[2]	ND[2]
13	0.0802[3] (0.617)	ND[2]	ND[2]	ND[2]
25	0.0475[3] (0.190)	ND[2]	ND[2]	ND[2]
50	ND[2]	ND[2]	ND[2]	ND[2]
100	2.33[3] (2.33)	ND[2]	ND[2]	ND[2]

[1] Results were generated using Microsoft Excel 2010 in full precision mode. Manual calculations may differ slightly.

[2] ND denotes not detected. The method limit of detection (LOD) was 0.0664 mg a.i./L.

[3] The reported value is extrapolated and falls below the method limit of quantitation (LOQ). The method LOQ for CAS# in freshwater AAP medium was set at 3.00 mg a.i./L , based upon the lowest analyte concentration in a fortified sample that obtained a mean recovery of 70-110%.

TABLE 2 QUALITY CONTROL SAMPLES OF CAS# IN FRESHWATER AAP MEDIUM

Sample Number (783P-102-)	Sample Type	Concentration of CAS# Fortified (mg a.i./L)	Concentration of CAS# Measured[1,2,3] (mg a.i./L)	Percent Recovery[2,3]
MAB-1	Matrix Blank	0.0	ND	--
MAB-2	Matrix Blank	0.0	ND	--
MAB-3	Matrix Blank	0.0	ND	--
MAB-4	Matrix Blank	0.0	ND	--
MAS-1	Matrix Fortification	3.00	2.81	93.6
MAS-2	Matrix Fortification	100	101	101
MAS-3	Matrix Fortification	3.00	2.62	87.2
MAS-4	Matrix Fortification	100	98.3	98.3
MAS-5	Matrix Fortification	3.00	2.79	93.1
MAS-6	Matrix Fortification	100	103	103
MAS-7	Matrix Fortification	3.00	2.67	89.1
MAS-8	Matrix Fortification	100	73.6	73.6
			Mean[3] =	92.4
			SD[3] =	9.37
			RSD[3] =	10.1%

[1] ND denotes not detected. The method limit of detection (LOD) was 0.0664 mg a.i./L.

[2] The method limit of quantitation (LOQ) for CAS# in freshwater AAP medium was set at 3.00 mg a.i./L, based upon the lowest analyte concentration in a fortified sample that obtained a mean recovery of 70-110%.

[3] Results were generated using Microsoft Excel 2010 in full precision mode. Manual calculations may differ slightly.

TABLE 3 TEMPERATURE MEASUREMENTS

Time (Days)	Temperature (°C)[1]		
	Minimum	Maximum	Mean
0	23.40	24.55	24.15
1	24.30	24.55	24.44
2	24.17	24.36	24.29
3	24.23	24.30	24.28
0-3	24.40	24.55	24.29

[1] Temperature was continuously monitored in a container of reverse-osmosis water located adjacent to the test with an Amegaview Scientific centralized monitoring system.

TABLE 4 pH MEASUREMENTS

Nominal Concentration (mg a.i./L)	pH Measurements	
	Day 0[1]	Day 3[2]
Blank Control	7.6	10.0
6.3	7.8	10.3
13	7.7	10.3
25	7.7	10.3
50	7.7	10.3
100	7.7	10.2

[1] Day 0 samples were collected from the individual batches of test solution prepared for each test concentration and blank control solution at test initiation.

[2] Day 3 samples were composites of test solution collected from each of the remaining replicates per test concentration and blank control.

TABLE 5 LIGHT INTENSITY MEASUREMENTS ON DAY 0

Shaker Table ID #	Light Intensity Measurements (lux)[1]				
AQL#2	5,610	5,590	5,860	6,190	6,420
AQL#1	6,150	6,000	5,680	5,440	5,830
AQL#3	5,900	5,680	5,620	6,230	6,060

[1] Measurements were taken at test initiation at five locations surrounding the test flasks on the shaker tables.

TABLE 6 MEAN CELL DENSITY, MEAN YIELD, AND PERCENT INHIBITION DURING THE 72-HOUR EXPOSURE

	24 Hours		48 Hours		72 Hours		0-72 Hours	
Nominal Concentration (mg a.i./L)	Mean Cell Density (cells/mL)	Percent Inhibition [1,2]	Mean Cell Density (cells/mL)	Percent Inhibition [1,2]	Mean Cell Density (cells/mL)*	Percent Inhibition [1,2]	Mean Yield (cells/mL)*	Percent Inhibition [1,2]
Blank Control	55,834	--	210,119	--	523,657	--	518,657	--
6.3	58,141	-4	211,205	-1	748,127	-43	743,127	-43
13	47,505	15	214,436	-2	683,072	-30	678,072	-31
25	49,525	11	202,832	3	811,022	-55	806,022	-55
50	42,803	23	207,666	1	654,198	-25	649,198	-25
100	44,680	20	209,297	0	677,350	-29	672,350	-30

[1] Calculations were performed using SAS Version 9.4. Manual calculations may differ slightly.

[2] Inhibition was calculated relative to the mean blank control response.

* None of the treatment group means were significantly reduced (Dunnett's test, $p > 0.05$) when compared to the blank control mean. Significance only evaluated for cell density at 72 hours of exposure.

TABLE 7 MEAN GROWTH RATE AND PERCENT INHIBITION DURING THE 72-HOUR EXPOSURE

Nominal Concentration (mg a.i./L)	0-24 Hours		0-48 Hours		0-72 Hours	
	Mean Growth Rate ($hour^{-1}$)[1]	Percent Inhibition [2]	Mean Growth Rate ($hour^{-1}$)[1]	Percent Inhibition [2]	Mean Growth Rate ($hour^{-1}$)[1,*]	Percent Inhibition [2]
Blank Control	0.0996	--	0.0779	--	0.0645	--
6.3	0.1020	-2	0.0779	0	0.0695	-8
13	0.0938	6	0.0780	0	0.0682	-6
25	0.0955	4	0.0770	1	0.0706	-10
50	0.0894	10	0.0776	0	0.0675	-5
100	0.0911	9	0.0778	0	0.0680	-5

[1] Calculations were performed using SAS Version 9.4. Manual calculations may differ slightly.
[2] Percent inhibition was calculated relative to the blank control replicates.
* None of the treatment group means were significantly reduced (Dunnett's test, $p > 0.05$) when compared to the blank control mean. Significance only evaluated at 72 hours of exposure.

TABLE 8 ECx, EyCx, AND ErCx VALUES FOR THE 72-HOUR EXPOSURE PERIOD

Duration of Exposure	Cell Density (mg a.i./L)		Yield (mg a.i./L)		Growth Rate (mg a.i./L)	
	EC_{50}	95% Confidence Interval	E_yC_{50}	95% Confidence Interval	E_rC_{50}	95% Confidence Interval
24 Hours	>100	n/a	--[1]	--[1]	>100	n/a
48 Hours	>100	n/a	--[1]	--[1]	>100	n/a
72 Hours	>100	n/a	>100	n/a	>100	n/a
	NOEC (mg a.i./L)		NOEC (mg a.i./L)		NOEC (mg a.i./L)	
72 Hours	100		100		100	

[1] E_yC_{50} values only determined at 72 hours per study protocol.

FIGURE 1 GROWTH OF *RAPHIDOCELIS SUBCAPITATA* IN THE BLANK CONTROL DURING THE 72-HOUR EXPOSURE PERIOD

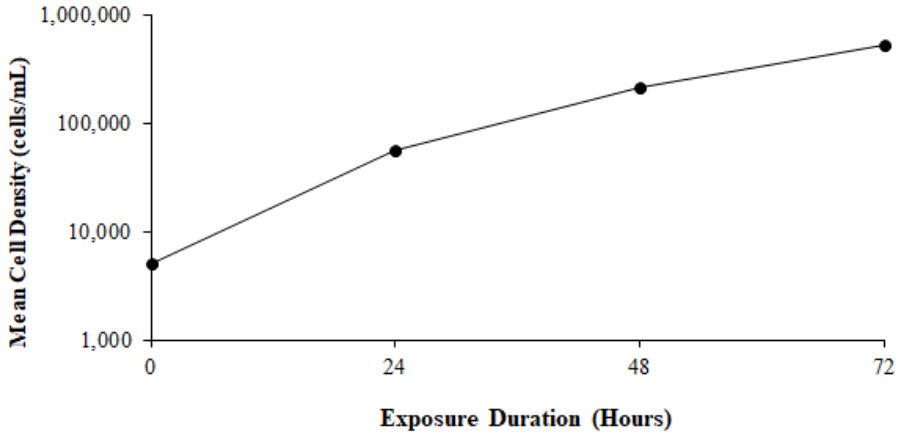
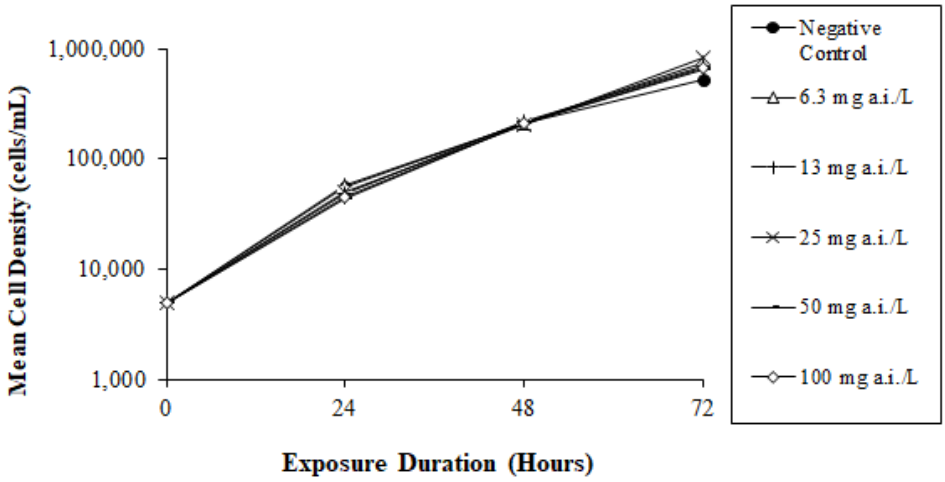


FIGURE 2 CELL DENSITY VERSUS TIME FOR *RAPHIDOCELIS SUBCAPITATA* EXPOSED TO CAS# FOR 72 HOURS



APPENDIX 1 CERTIFICATE OF ANALYSIS

APPENDIX 2 FRESHWATER ALGAL (AAP) MEDIUM

Component	Nominal Concentration
$MgCl_2 \cdot 6H_2O$	12.164 mg/L
$CaCl_2 \cdot 2H_2O$	4.410 mg/L
H_3BO_3	0.1855 mg/L
$MnCl_2 \cdot 4H_2O$	0.4154 mg/L
$ZnCl_2$	3.27 μg/L
$FeCl_3 \cdot 6H_2O$	0.1598 mg/L
$CoCl_2 \cdot 6H_2O$	1.428 μg/L
$Na_2MoO_4 \cdot 2H_2O$	7.26 μg/L
$CuCl_2 \cdot 2H_2O$	0.012 μg/L
$Na_2EDTA \cdot 2H_2O$	0.300 mg/L
$NaNO_3$	25.50 mg/L
$MgSO_4 \cdot 7H_2O$	14.70 mg/L
K_2HPO_4	1.044 mg/L
$NaHCO_3$	50.00 mg/L*

The pH of the medium was adjusted to 7.5 ± 0.1 with 10% HCl, as necessary.

*The test medium contained an additional 35 mg/L of sodium bicarbonate due to the closed bottle test design.

APPENDIX 3 ANALYSES OF PESTICIDES, ORGANICS AND METALS IN EAG LABORATORIES -- EASTON WELL WATER

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Analysis Report

Sample Description: Well Water
2017 Facility Samples

Project Name: **2017 Facility Samples**

Submittal Date/Time: 12/21/2017 11:55
Collection Date/Time: 12/20/2017

Wildlife International,
ELLE Sample #: WW 9381758
ELLE Group #: 1889983
Matrix: Wastewater

CAT No.	Analysis Name	CAS Number	Result	Limit of Quantitation	Dilution Factor
Pesticides	**SW-846 8081A**		ug/l	ug/l	
00177	Aldrin	309-00-2	< 0.0092 D1	0.0092	1
00177	Alpha BHC	319-84-6	< 0.0092 D2	0.0092	1
00177	Beta BHC	319-85-7	< 0.0092 D2	0.0092	1
00177	Gamma BHC - Lindane	58-89-9	< 0.0092 D1	0.0092	1
00177	Alpha Chlordane	5103-71-9	< 0.0092 D2	0.0092	1
00177	Chlordane	57-74-9	< 0.46 D2	0.46	1
00177	Gamma Chlordane	5103-74-2	< 0.018 D1	0.018	1
00177	o,p-DDD	53-19-0	< 0.018 D1	0.018	1
00177	p,p-DDD	72-54-8	< 0.018 D1	0.018	1
00177	o,p-DDE	3424-82-6	< 0.018 D1	0.018	1
00177	p,p-DDE	72-55-9	< 0.018 D1	0.018	1
00177	o,p-DDT	789-02-6	< 0.018 D1	0.018	1
00177	p,p-DDT	50-29-3	< 0.018 D1	0.018	1
00177	Delta BHC	319-86-8	< 0.0092 D2	0.0092	1
00177	Dieldrin	60-57-1	< 0.018 D1	0.018	1
00177	Endosulfan I	959-98-8	< 0.0092 D2	0.0092	1
00177	Endosulfan II	33213-65-9	< 0.028 D1	0.028	1
00177	Endosulfan Sulfate	1031-07-8	< 0.018 D1	0.018	1
00177	Endrin	72-20-8	< 0.018 D1	0.018	1
00177	Endrin Aldehyde	7421-93-4	< 0.092 D1	0.092	1
00177	Endrin Ketone	53494-70-5	< 0.018 D1	0.018	1
00177	HCB	118-74-1	< 0.0092 D1	0.0092	1
00177	Heptachlor	76-44-8	< 0.0092 D1	0.0092	1
00177	Heptachlor Epoxide	1024-57-3	< 0.0092 D1	0.0092	1
00177	Kepone	143-50-0	< 0.18 ZD1	0.18	1
00177	Methoxychlor	72-43-5	< 0.092 D1	0.092	1
00177	Mirex	2385-85-5	< 0.046 D2	0.046	1
00177	Telodrin	297-78-9	< 0.0092 D1	0.0092	1
00177	Toxaphene	8001-35-2	< 0.92 D1	0.92	1

Z=The % difference for the calibration verification standard is outside the +/- 15% criteria. Since the average of the% difference values meets the criteria, the results are reported.

Pesticides	**SW-846 8141A**		ug/l	ug/l	
10410	Bolstar	35400-43-2	< 5.2 D1	5.2	1
10410	Coumaphos	56-72-4	< 5.2 D1	5.2	1
10410	Demeton-O	298-03-3	< 5.2 D1	5.2	1
10410	Demeton-S	126-75-0	< 5.2 D1	5.2	1
10410	Diazinon	333-41-5	< 5.2 D1	5.2	1
10410	Dichlorvos	62-73-7	< 5.2 D1	5.2	1
10410	Disulfoton	298-04-4	< 5.2 D1	5.2	1
10410	Dursban (Chlorpyrifos)	2921-88-2	< 5.2 D1	5.2	1
10410	EPN	2104-64-5	< 5.2 D1	5.2	1
10410	Ethion	563-12-2	< 5.2 D1	5.2	1
10410	Ethoprop	13194-48-4	< 5.2 D1	5.2	1
10410	Ethyl Parathion	56-38-2	< 5.2 D1	5.2	1
10410	Famphur	52-85-7	< 5.2 D1	5.2	1
10410	Fensulfothion	115-90-2	< 7.9 D1	7.9	1
10410	Fenthion	55-38-9	< 5.2 D1	5.2	1
10410	Guthion (Azinphos-methyl)	86-50-0	< 5.2 D1	5.2	1
10410	Malathion	121-75-5	< 5.2 D1	5.2	1

APPENDIX 3 ANALYSES OF PESTICIDES, ORGANICS AND METALS IN EAG LABORATORIES -- EASTON WELL WATER (CONTINUED)

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Analysis Report

Sample Description: Well Water
2017 Facility Samples

Project Name: 2017 Facility Samples

Submittal Date/Time: 12/21/2017 11:55
Collection Date/Time: 12/20/2017

Wildlife International,
ELLE Sample #: WW 9381758
ELLE Group #: 1889983
Matrix: Wastewater

CAT No.	Analysis Name	CAS Number	Result	Limit of Quantitation	Dilution Factor
Pesticides		**SW-846 8141A**	ug/l	ug/l	
10410	Merphos	150-50-5	< 5.2 D1	5.2	1
10410	Methyl Parathion	298-00-0	< 5.2 D1	5.2	1
10410	Mevinphos	7786-34-7	< 5.2 D1	5.2	1
10410	Naled	300-76-5	< 5.2 D1	5.2	1
10410	Phorate	298-02-2	< 5.2 D1	5.2	1
10410	Ronnel	299-84-3	< 5.2 D1	5.2	1
10410	Stirofos	961-11-5	< 5.2 D1	5.2	1
10410	Tokuthion	34643-46-4	< 5.2 D1	5.2	1
10410	Trichloronate	327-98-0	< 5.2 D1	5.2	1
10410	Trithion	786-19-6	< 5.2 D1	5.2	1
Metals		**EPA 200.7 rev 4.4**	mg/l	mg/l	
01743	Aluminum	7429-90-5	< 0.200	0.200	1
07044	Antimony	7440-36-0	< 0.0200	0.0200	1
07035	Arsenic	7440-38-2	< 0.0200	0.0200	1
07046	Barium	7440-39-3	< 0.0050	0.0050	1
07047	Beryllium	7440-41-7	< 0.0050	0.0050	1
07049	Cadmium	7440-43-9	< 0.0050	0.0050	1
01750	Calcium	7440-70-2	36.2	0.200	1
07051	Chromium	7440-47-3	< 0.0150	0.0150	1
07052	Cobalt	7440-48-4	< 0.0050	0.0050	1
07053	Copper	7440-50-8	< 0.0100	0.0100	1
01754	Iron	7439-89-6	< 0.200	0.200	1
07055	Lead	7439-92-1	< 0.0150	0.0150	1
01757	Magnesium	7439-95-4	14.0	0.100	1
07058	Manganese	7439-96-5	< 0.0050	0.0050	1
07061	Nickel	7440-02-0	< 0.0100	0.0100	1
01762	Potassium	7440-09-7	7.06	0.500	1
07036	Selenium	7782-49-2	< 0.0200	0.0200	1
07066	Silver	7440-22-4	< 0.0050	0.0050	1
01767	Sodium	7440-23-5	18.1	1.00	1
07022	Thallium	7440-28-0	< 0.0300	0.0300	1
07071	Vanadium	7440-62-2	< 0.0050	0.0050	1
07072	Zinc	7440-66-6	< 0.0200	0.0200	1
		EPA 245.1 rev 3	mg/l	mg/l	
00259	Mercury	7439-97-6	< 0.00020	0.00020	1
Wet Chemistry		**EPA 300.0**	mg/l	mg/l	
01505	Bromide	24959-67-9	< 2.5	2.5	5
00224	Chloride	16887-00-6	4.3	2.0	5
01504	Fluoride	16984-48-8	< 0.50	0.50	5
00368	Nitrate Nitrogen	14797-55-8	< 0.50	0.50	5
01506	Nitrite Nitrogen	14797-65-0	< 0.50	0.50	5
00228	Sulfate	14808-79-8	5.5	5.0	5

Sample Comments

All QC is compliant unless otherwise noted. Please refer to the Quality Control Summary for overall QC performance data and associated samples.

APPENDIX 3 ANALYSES OF PESTICIDES, ORGANICS AND METALS IN EAG LABORATORIES -- EASTON WELL WATER (CONTINUED)

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2425 New Holland Pike, Lancaster, PA 17601 • 717-656-2300 • Fax: 717-656-6766 • www.EurofinsUS.com/LancLabsEnv

Analysis Report

Sample Description: Well Water
2017 Facility Samples

Project Name: 2017 Facility Samples

Submittal Date/Time: 12/21/2017 11:55
Collection Date/Time: 12/20/2017

Wildlife International,
ELLE Sample #: WW 9381758
ELLE Group #: 1889983
Matrix: Wastewater

Laboratory Sample Analysis Record

CAT No.	Analysis Name	Method	Trial#	Batch#	Analysis Date and Time	Analyst	Dilution Factor
00177	OC Pesticides in Water	SW-846 8081A	1	173610004A	01/09/2018 23:08	Heather E Williams	1
10410	OP Pest water 8141A Master	SW-846 8141A	1	173610007A	01/05/2018 23:34	Richard A Shober	1
06854	OP Pest Water Ext. (Turbovap)	SW-846 3510C	1	173610007A	12/27/2017 18:54	Kate E Lutte	1
11118	Pesticide Screen Waters Ext	SW-846 3510C	1	173610004A	12/27/2017 18:54	Kate E Lutte	1
01743	Aluminum	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07044	Antimony	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07035	Arsenic	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07046	Barium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07047	Beryllium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07049	Cadmium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
01750	Calcium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07051	Chromium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07052	Cobalt	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07053	Copper	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
01754	Iron	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07055	Lead	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
01757	Magnesium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07058	Manganese	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07061	Nickel	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
01762	Potassium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07036	Selenium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07066	Silver	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
01767	Sodium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07022	Thallium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07071	Vanadium	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
07072	Zinc	EPA 200.7 rev 4.4	1	173560571610	01/11/2018 04:30	Jonathan J Allen	1
00259	Mercury	EPA 245.1 rev 3	1	173560571404	12/27/2017 09:47	Damary Valentin	1
05716	EPA 600 ICP Digest (tot rec)	EPA 200.7 rev 4.4	1	173560571610	12/27/2017 07:15	Lisa J Cooke	1
05714	PW/WW Hg Digest	EPA 245.1 rev 3	1	173560571404	12/27/2017 01:05	Denise L Trimby	1
01505	Bromide	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5
00224	Chloride	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5
01504	Fluoride	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5
00368	Nitrate Nitrogen	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5
01506	Nitrite Nitrogen	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5
00228	Sulfate	EPA 300.0	1	17355249117A	12/21/2017 21:29	Zachary W Enck	5

APPENDIX 3 ANALYSES OF PESTICIDES, ORGANICS AND METALS IN EAG LABORATORIES -- EASTON WELL WATER (CONTINUED)

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Data Qualifiers

Qualifier	Definition
C	Result confirmed by reanalysis
D1	Indicates for dual column analyses that the result is reported from column 1
D2	Indicates for dual column analyses that the result is reported from column 2
E	Concentration exceeds the calibration range
J (or G, I, X)	Estimated value >= the Method Detection Limit (MDL or DL) and < the Limit of Quantitation (LOQ or RL)
P	Concentration difference between the primary and confirmation column >40%. The lower result is reported.
U	Analyte was not detected at the value indicated
V	Concentration difference between the primary and confirmation column >100%. The reporting limit is raised due to this disparity and evident interference.
W	The dissolved oxygen uptake for the unseeded blank is greater than 0.20 mg/L.
Z	Laboratory Defined - see analysis report

Additional Organic and Inorganic CLP qualifiers may be used with Form 1 reports as defined by the CLP methods.
Qualifiers specific to Dioxin/Furans and PCB Congeners are detailed on the individual Analysis Report.

APPENDIX 4 CHANGES TO THE STUDY PROTOCOL

This study was conducted in accordance with the approved protocol with the exception of the following deviation:

1. Only 5 replicates remained for the blank control group at the 72-hour sampling interval. This deviation from the approved protocol was not considered to be detrimental to the study.

APPENDIX 5 ANALYSIS OF CAS# IN FRESHWATER AAP MEDIUM

APPENDIX 5, FIGURE 1 ANALYTICAL METHOD FLOWCHART FOR THE ANALYSIS OF CAS# IN FRESHWATER AAP MEDIUM

1. Prepare calibration standards in Toluene from a stock solution prepared in acetone. Transfer aliquots of each into autosampler vials for analysis. Transfer remaining to scintillation vials with Teflon lined caps.

2. Pre-weigh sodium chloride for each sample into weigh boats.

3. Study samples will have the appropriate volume of toluene added when collected by aquatics laboratory. Add 1.0g of sodium chloride to each sample, before vortexing each sample for approximately 1 minute each using a hand vortexer.

4. Centrifuge samples at approximately 491 RCF for approximately 5 minutes in order to separate the aqueous and organic layers. Then, carefully remove an aliquot of each upper toluene layer and transfer it to an autosampler vial for analysis. Transfer the remaining toluene layers into scintillation vials with Teflon lined caps.

5. Lastly, prepare quality control (QC) samples in glass scintillation vials with Teflon lined caps.

    a. Pre-weigh sodium chloride for each QC sample into weigh boats.

    b. Add freshwater AAP medium to each vial, and remove the appropriate fortification volume using a pipettor, or equivalent.

    c. To fortify each sample, use a gastight syringe to add the aliquot of the appropriate stock approximately 2/3 of the way beneath the water surface. The matrix blank will be unfortified freshwater AAP medium.

    d. Add toluene to each matrix fortification sample immediately after fortifying.

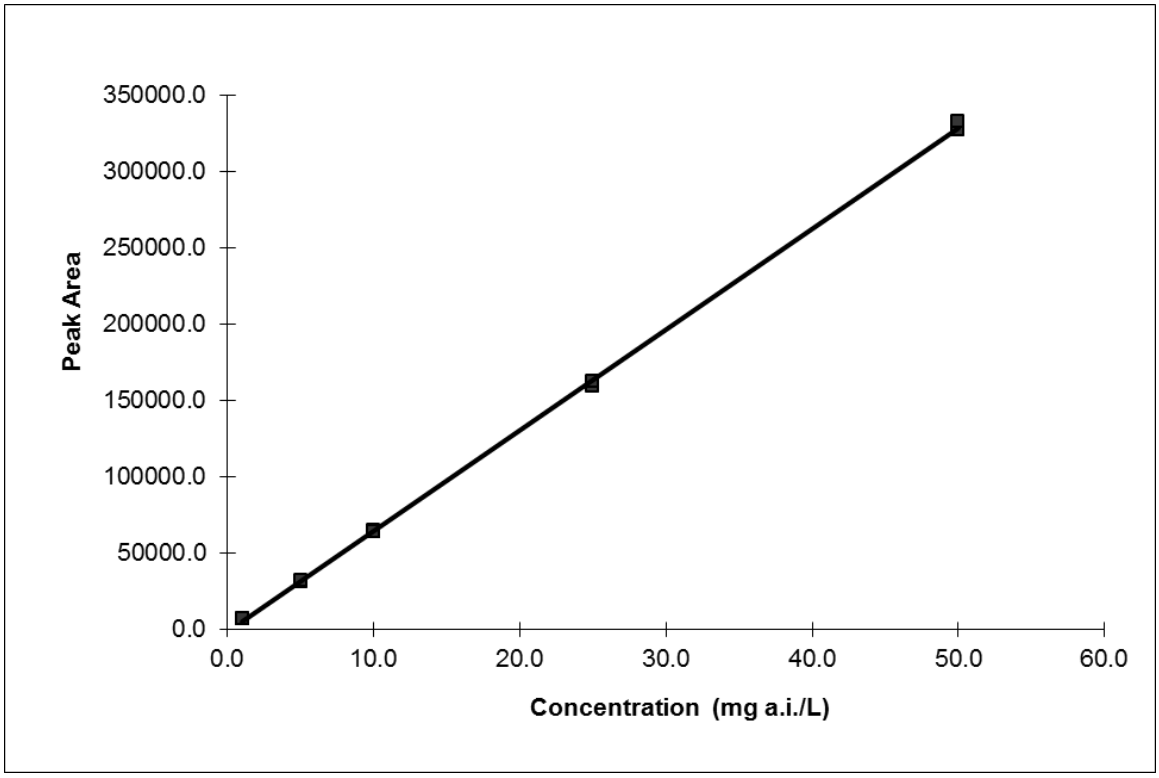
    e. Then, immediately add the pre-weighed sodium chloride, cap and invert a few times to mix.

6. Once all QC samples are prepared, vortex each sample for approximately 1 minute each using a hand vortexer.

7. Centrifuge samples at approximately 491 RCF for approximately 5 minutes in order to separate the aqueous and organic layers. Then, carefully remove an aliquot of each upper toluene layer and transfer it to an autosampler vial for analysis. Transfer the remaining toluene layers into scintillation vials with Teflon lined caps.

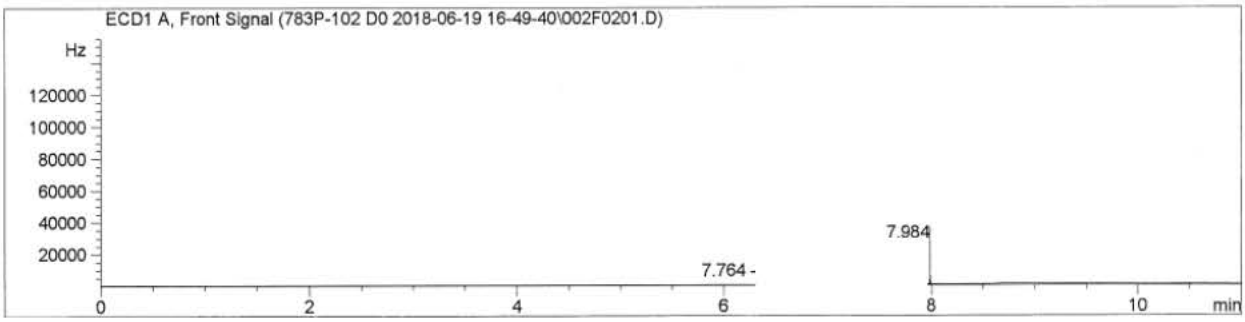
8. Submit standards and samples for analysis by GC-ECD.

APPENDIX 5, FIGURE 2 REPRESENTATIVE CALIBRATION CURVE FOR CAS#



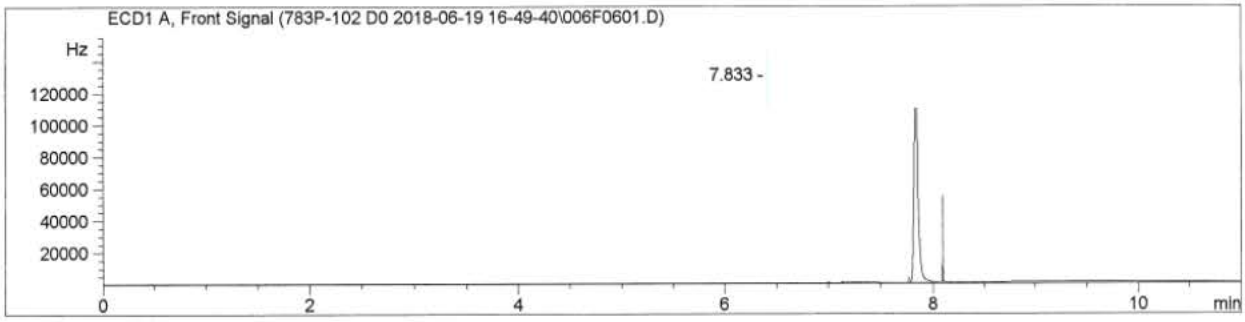
y-intercept = 256; Slope = 6514; R^2 = 0.99957

APPENDIX 5, FIGURE 3 REPRESENTATIVE CHROMATOGRAMS OF A LOW-LEVEL CALIBRATION STANDARD FOR CAS#



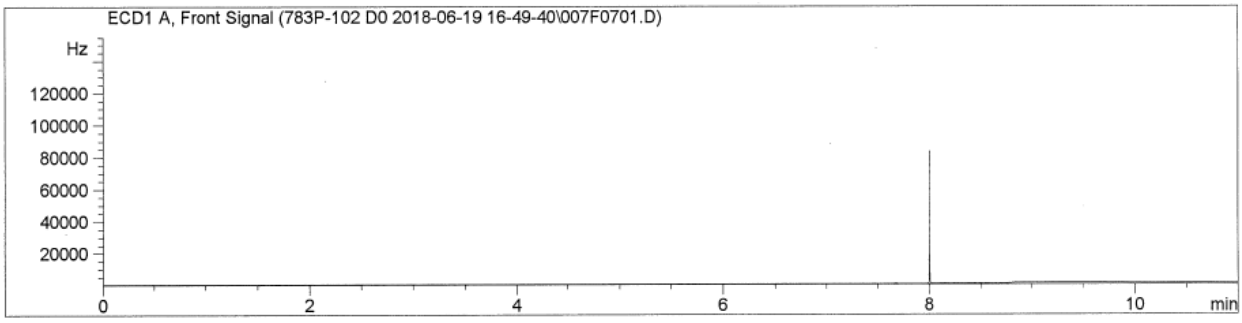
Calibration standard solution contains CAS# at a concentration of 1.00 mg a.i./L. CAS# elutes at a retention time of approximately 7.8 minutes.

APPENDIX 5, FIGURE 4 REPRESENTATIVE CHROMATOGRAMS OF A HIGH-LEVEL CALIBRATION STANDARD FOR CAS#



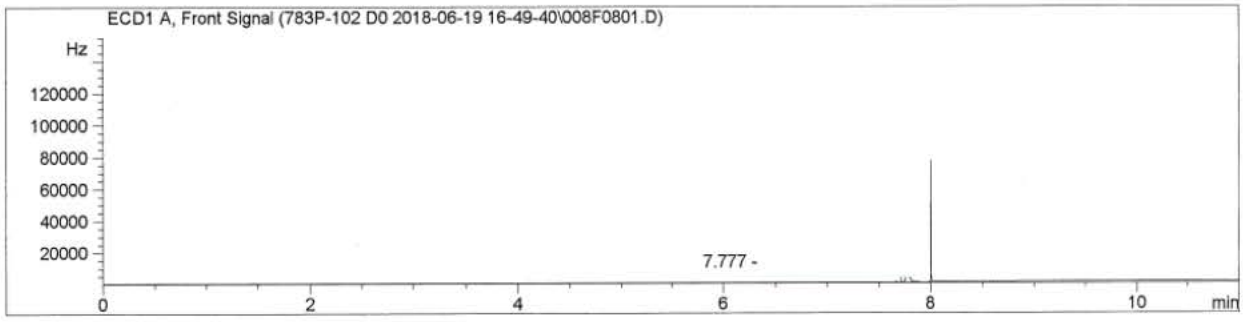
Calibration standard solution contains CAS# at a concentration of 50.0 mg a.i./L. CAS# elutes at a retention time of approximately 7.8 minutes.

APPENDIX 5, FIGURE 5 REPRESENTATIVE CHROMATOGRAMS OF A MATRIX BLANK SAMPLE



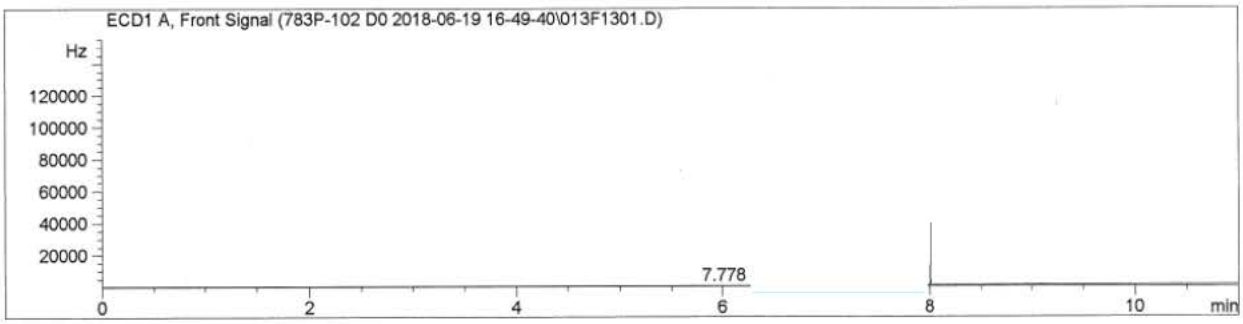
CAS# elutes at a retention time of approximately 7.8 minutes. (Sample 783P-102-MAB-1).

APPENDIX 5, FIGURE 6 REPRESENTATIVE CHROMATOGRAMS OF A MATRIX FORTIFICATION SAMPLE ANALYZED FOR CAS#



Matrix fortification sample (783P-102-MAS-1) contains CAS# at a nominal concentration of 3.00 mg a.i./L. CAS# elutes at a retention time of approximately 7.8 minutes.

APPENDIX 5, FIGURE 7 REPRESENTATIVE CHROMATOGRAMS OF A TEST SAMPLE ANALYZED FOR CAS# IN FRESHWATER AAP MEDIUM



Test solution sample (783P-102-3) contains CAS# at a nominal concentration of 13 mg a.i./L. CAS# elutes at a retention time of approximately 7.8 minutes.

APPENDIX 6 CELL DENSITY AND YIELD BY REPLICATE

Nominal Test Concentration (mg a.i./L)	Replicate	Cell Density (cells/mL)[1]			Yield (cells/mL)[2]
		24 Hours	48 Hours	72 Hours	72 Hours
Blank Control	A	54,314	212,912	578,023	573,023
	B	55,856	229,986	542,347	537,347
	C	42,616	211,273	--*	--*
	D	48,119	200,310	414,611	409,611
	E	51,989	203,959	530,926	525,926
	F	82,110	202,275	552,377	547,377
6.3	A	51,120	237,976	792,232	787,232
	B	64,685	184,804	751,606	746,606
	C	58,617	210,834	700,542	695,542
13	A	46,691	263,209	710,851	705,851
	B	49,140	177,442	601,516	596,516
	C	46,685	202,658	736,848	731,848
25	A	52,337	214,665	781,883	776,883
	B	47,329	175,315	763,138	758,138
	C	48,909	218,516	888,045	883,045
50	A	40,499	221,478	646,213	641,213
	B	45,192	185,208	773,916	768,916
	C	42,717	216,311	542,464	537,464
100	A	46,294	202,753	849,657	844,657
	B	47,849	199,488	602,095	597,095
	C	39,897	225,651	580,299	575,299

[1] The initial cell density of the stock culture was determined and an inoculum volume was administered to each test chamber to yield a cell density of approximately 5,000 cells/mL at test initiation (0 hours).

[2] Yield was calculated as the final cell density minus the nominal initial cell density (5,000 cells/mL).

* Only 5 replicate test vessels remained at the end of the study in the blank control.

APPENDIX 7 GROWTH RATE BY REPLICATE

Nominal Test Concentration (mg a.i./L)	Replicate	Growth Rate (per hour)		
		0 - 24 Hours	0 – 48 Hours	0 – 72 Hours
Blank Control	A	0.0994	0.0782	0.0660
	B	0.1006	0.0798	0.0651
	C	0.0893	0.0780	--*
	D	0.0943	0.0769	0.0614
	E	0.0976	0.0773	0.0648
	F	0.1166	0.0771	0.0653
6.3	A	0.0969	0.0805	0.0704
	B	0.1067	0.0752	0.0696
	C	0.1026	0.0780	0.0686
13	A	0.0931	0.0826	0.0688
	B	0.0952	0.0744	0.0665
	C	0.0931	0.0771	0.0693
25	A	0.0978	0.0783	0.0702
	B	0.0937	0.0741	0.0698
	C	0.0950	0.0787	0.0719
50	A	0.0872	0.0790	0.0675
	B	0.0917	0.0753	0.0700
	C	0.0894	0.0785	0.0651
100	A	0.0927	0.0771	0.0713
	B	0.0941	0.0768	0.0665
	C	0.0865	0.0794	0.0660

* Only 5 replicate test vessels remained at the end of the study in the blank control.

APPENDIX 8 SPECIFIC GROWTH RATE OF THE BLANK CONTROL GROUP

Section-by-Section Growth Rate for Blank Control

Control Replicate	0-24 Hour Growth Rate	24-48 Hour Growth Rate	48-72 Hour Growth Rate	% COEFFICIENT OF VARIATION[1]
A	0.0994	0.0569	0.0416	45.4
B	0.1006	0.0590	0.0357	50.5
C	--*	--*	--*	--*
D	0.0943	0.0594	0.0303	52.3
E	0.0976	0.0570	0.0399	45.7
F	0.1166	0.0376	0.0419	68.0
			Mean:	52.4

[1]Coefficient of Variation = S.D./mean *100
Results were generated using Excel 2010 in the full precision mode. Manual calculations may differ slightly.
*Only 5 replicate test vessels remained at the end of the study in the blank control.

Average Specific Growth Rate During the Whole Test Period

Control Replicate	0-72 Hour Growth Rate
A	0.0660
B	0.0651
C	--*
D	0.0614
E	0.0648
F	0.0653
% Coefficient of Variation:	2.8

Results were generated using Excel 2010 in the full precision mode. Manual calculations may differ slightly.
*Only 5 replicate test vessels remained at the end of the study in the blank control.

APPENDIX 9 PERSONNEL INVOLVED IN THE STUDY

The following individuals from EAG Laboratories participated in the conduct of this study:

Study Director:	Joshua R. Arnie, B.S.
Management:	John A. Aufderheide, B.A. Kathy H. Martin, M.S.
Supervisory Personnel:	John A. Aufderheide, B.A. Kathy H. Martin, M.S.
Biologists:	Joshua R. Arnie, B.S. Chelsey N. Tull, B.S.
Analysis of Test Solutions:	Laura A. Lockard, M.S.
Report Preparation:	Joshua R.Arnie, B.S. Laura A. Lockard, M.S.
Sponsor Study Monitor:	

APPENDIX 10 DETAILED STUDY SUMMARY

Freshwater Alga (*Raphidocelis subcapitata*)

CAS# A 72-Hour Toxicity Test with the Freshwater Alga (*Raphidocelis subcapitata*)

Report authors: Joshua R. Arnie, B.S.; Laura A. Lockard, M.S.; John A. Aufderheide, B.S.; Kathy H. Martin, M.S.

Testing facility: EAG Laboratories, 8598 Commerce Drive, Easton, Maryland 21601, U.S.A.

Executive summary:

The effect of CAS# on the cell density, growth rate, and yield of the freshwater alga, *Raphidocelis subcapitata* was determined in a 72-hour test without test medium renewal. The test was conducted according to OECD Guidelines for the Testing of Chemicals: 201 (2011). Treatments consisted of five nominal CAS# concentrations of 6.3, 13, 25, 50, and 100 mg a.i./L and an untreated blank control. Samples were collected for analytical verification at 0, 24, 48, and 72 hours of exposure. Measured concentrations of CAS# in samples collected from the treatment groups at all sampling intervals were <LOQ. Due to the volatile nature of the test substance, the reported density was used to calculate the volume of test substance to be used in each test solution and Hamilton gas-tight syringes were used to measure the appropriate volume. Additionally, the mixing vessels and test chambers were closed vessels with no headspace. Replicates included for cell density determinations and analytical sampling were removed from the study after sampling. The EC50 and NOEC values for *R. subcapitata* were based on nominal measured concentrations of CAS# for cell density, growth rate, and yield. The 72-hour EC50, E_rC50, and E_yC50 values based on cell density, growth rate, and yield, respectively, were >100 mg a.i./L, for all three endpoints. The NOEC for all three endpoints was determined to be 100 mg a.i./L..

I. MATERIALS AND METHODS

A. MATERIALS

1.	Test material:	CAS#
	Lot #:	Sample# 12639
	Purity:	
	Description:	Liquid
	Stability of test compound:	N/A
2.	Controls:	Freshwater algal (AAP) medium
	Test vehicle:	None
	Toxic reference:	None
3.	Test organism:	Freshwater Alga
	Species:	*Raphidocelis subcapitata*
	Initial population:	Approximately 5,000 cells/mL
	Source:	EAG Laboratories, Easton, MD in-house culture
	Growth medium:	Freshwater algal (AAP) medium
	Test chamber:	300-mL glass bottles sealed with glass stoppers filled with no headspace
4.	Environmental conditions:	
	Temperature:	24.40 to 24.55°C (measured in a container of water located adjacent to the test)
	Photoperiod:	24-hour photoperiod (5,440 to 6,420 lux)
	pH	7.6 to 10.3 throughout the exposure period

B. STUDY DESIGN AND METHODS

1. In-life initiated/completed

December 18 to December 21, 2017

2. Experimental treatments

A study was conducted to determine the effect of CAS# on the cell density, growth rate, and yield of the freshwater alga *Raphidocelis subcapitata*. The algae were exposed to an untreated blank control and five nominal test substance concentrations of 6.3, 13, 25, 50, and 100 mg a.i./L, in freshwater AAP medium for 72 hours, without test medium renewal. Six replicate test chambers in the control group and three replicate test chambers in each treatment (test concentration) group were included for each 24-hour sampling interval. CAS# is volatile, therefore, the study was conducted in a closed system in an attempt to maintain exposure concentrations. There were a total of nine replicates in each treatment group and 18 blank control replicates at test initiation.

3. Observations

Cell counts were recorded for samples collected approximately 24, 48, and 72 hours after test initiation. Cell densities, growth rates and yield were calculated and expressed as percent inhibition relative to the blank control replicates following exposure to CAS# for 72 hours.

4. Statistics

The 72-hour cell density, growth rate, and yield data were evaluated for normality and homogeneity of variance ($\alpha = 0.01$) using Shapiro-Wilk's and Levene's tests, respectively. All data met assumptions of normality and homogeneity of variance. The mean treatment group responses were compared to the blank control response using Dunnett's one-tailed t-test ($\alpha = 0.05$). The results of the statistical analyses of the cell density, growth rate, and yield data, as well as an evaluation of the concentration-response pattern, were used to determine the NOEC relative to each parameter at 72 hours.

II. RESULTS AND DISCUSSION

A. FINDINGS

Measured concentrations of CAS# in samples collected from the treatment groups at all sampling intervals were <LOQ. Due to the volatile nature of the test substance, the reported density was used to calculate the volume of test substance to be used in each test solution and Hamilton gas-tight syringes were used to measure the appropriate volume. Additionally, the mixing vessels and test chambers were closed vessels with no headspace. Replicates included for cell density determinations and analytical sampling were removed from the study after sampling. A summary of algal growth inhibition following exposure of *R. subcapitata* to CAS# for 72-hours is presented in the table that follows. The relevance of the OECD 201 test acceptance criteria to this study design where replicates are sacrificed every 24 hours is questionable. Cell density increased in the blank control by a factor of 105 in 72 hours. The coefficient of variation of average specific growth rates during the whole test period (0-72 hour) in replicates of the blank control was 2.8%. In addition, the mean percent coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, and 2-3) was 52.4%, which exceeded the OECD 201 requirement of 35%. This study employed a closed-bottle test design where replicates were sampled every 24 hours and after sampling they were removed from the study. This particular criterion should not be considered applicable following this test design since its impossible to monitor growth in the control replicates over time.

Table 1

Table 1. Summary of algal growth inhibition following exposure of *Raphidocelis subcapitata* to CAS# for 72 hours

Nominal Concentration (mg a.i./L)	% Inhibition Relative to Blank Control		
	Cell Density*	Growth rate*	Yield*
Blank Control (0.0)	—	—	—
0.0023	-43	-8	-43
0.0087	-30	-6	-31
0.030	-55	-10	-55
0.097	-25	-5	-25
0.31	-29	-5	-30

* None of the treatment group means were significantly different from the blank control mean (Dunnett's test, $p > 0.05$).

III. CONCLUSION

The effects of CAS# on cell density, growth rate and yield of *Raphidocelis subcapitata* as calculated using nominal test concentrations, were as follows:

	Nominal Test Concentration
Yield:	72-hr E_yC_{50} > 100 mg a.i./L (n/a) 72-hr NOEC = 100 mg a.i./L
Growth Rate:	72-hr E_rC_{50} > 100 mg a.i./L (n/a) 72-hr NOEC = 100 mg a.i./L
Cell Density:	72-hr EC_{50} > 100 mg a.i./L (n/a) 72-hr NOEC = 100 mg a.i./L

(Arnie, J.R., 2018)

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

Author

Emily Dakoulas, BS

Study Completion Date

21 June 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF25CY.503.BTL

Sponsor

Sponsor Number

1. STATEMENT OF COMPLIANCE

Study No. AF25CY.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The stability to define the test substance has not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

21 JUN 2018
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF25CY.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
05-Apr-2018	05-Apr-2018	Strain Characterization	05-Apr-2018	05-Apr-2018
05-Apr-2018	05-Apr-2018	Preparation of S9 Mixture	05-Apr-2018	05-Apr-2018
05-Apr-2018	09-Apr-2018	Protocol Review	17-May-2018	17-May-2018
16-May-2018	17-May-2018	Data/Draft Report	17-May-2018	17-May-2018
17-May-2018	17-May-2018	Protocol Amendment Review	17-May-2018	17-May-2018
12-Jun-2018	12-Jun-2018	Final Report	12-Jun-2018	12-Jun-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Lisa AnnMarie Fleshman 21-Jun-2018 1:34 pm GMT

Reason for signature: QA Approval

Printed by:Lisa AnnMarie Fleshman
Printed on:21-Jun-18

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4. STUDY INFORMATION

Study Conduct

Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF25CY.503.BTL
Sponsor No.:	

Test Substance

Identification:	
Batch No.:	12639
Purity:	99.09% IVE (per Certificate of Analysis)
Description:	Clear colorless liquid
Storage Conditions:	Room temperature, protected from light and under inert gas (nitrogen)
Receipt Date:	14 March 2018

Study Dates

Study Initiation Date:	03 April 2018
Experimental Starting Date (first day of data collection):	04 April 2018
Experimental Start Date (first day test substance administered to test system):	05 April 2018
Experimental Completion Date:	23 April 2018

Key Personnel

Study Director:	Emily Dakoulas, BS
Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Acetone was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

In the confirmatory mutagenicity assay, the dose levels tested were 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The Sponsor has determined the identity, strength, purity and composition or other characteristics to define the test substance. A copy of the Certificate of Analysis is included in Appendix III. The stability to define the test substance has not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization, using the following information.

The vehicle used to deliver to the test system was acetone.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Acetone	67-64-1	Sigma-Aldrich	SHBJ2689	99.72%	Jun 2019

Sponsor's instructions were used while weighing the test substance for the assay. The cylinder was fixed in a vertical position inside a glove box with the needle valve directed downward. The needle valve was carefully opened to allow the low viscosity liquid drip into the vessel. The required quantity was measured into the glass vessel. After the transfer was complete, the nipple was removed and rinsed with acetone. Consequently, a stream of nitrogen was directed at the cylinder fitting to flush away Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

For submission to Japanese regulatory agencies, additional information is included in Appendix IV.

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Acetone was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in acetone at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3880, Exp. Date: 31 Oct 2019) was purchased commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and six dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	1.4	1.0	1.6	1.7	2.6
B2	1.8	1.1	1.2	1.4	3.2

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in acetone are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

Neither precipitate nor toxicity was observed.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

A copy of the Common Technical Document Tables is included in Appendix V.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study,
did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF25CY.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF25CY
Date Plated: 4/5/2018
Evaluation Period: 4/9/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98		5000 µg	*15*	*6*	*1.1*	11[A], 19[A]
		1500 µg	*15*	*1*	*1.1*	15[A], 14[A]
		500 µg	*15*	*1*	*1.1*	15[A], 14[A]
		150 µg	*21*	*5*	*1.5*	17[A], 24[A]
		50.0 µg	*16*	*0*	*1.1*	16[A], 16[A]
		15.0 µg	*15*	*1*	*1.1*	15[A], 14[A]
		5.00 µg	*22*	*6*	*1.6*	17[A], 26[A]
		1.50 µg	*11*	*6*	*0.8*	7[A], 15[A]
	Acetone	100 µL	*14*	*4*		11[A], 17[A]
TA100		5000 µg	*93*	*14*	*1.3*	103[A], 83[A]
		1500 µg	*88*	*3*	*1.2*	86[A], 90[A]
		500 µg	*86*	*9*	*1.2*	79[A], 92[A]
		150 µg	*105*	*0*	*1.5*	105[A], 105[A]
		50.0 µg	*84*	*35*	*1.2*	108[A], 59[A]
		15.0 µg	*79*	*6*	*1.1*	74[A], 83[A]
		5.00 µg	*91*	*12*	*1.3*	82[A], 99[A]
		1.50 µg	*84*	*4*	*1.2*	81[A], 86[A]
	Acetone	100 µL	*72*	*11*		64[A], 80[A]
TA1535		5000 µg	*15*	*1*	*1.3*	16[A], 14[A]
		1500 µg	*18*	*1*	*1.5*	18[A], 17[A]
		500 µg	*16*	*1*	*1.3*	15[A], 17[A]
		150 µg	*10*	*2*	*0.8*	8[A], 11[A]
		50.0 µg	*17*	*4*	*1.4*	14[A], 19[A]
		15.0 µg	*9*	*1*	*0.8*	10[A], 8[A]
		5.00 µg	*10*	*1*	*0.8*	11[A], 9[A]
		1.50 µg	*14*	*1*	*1.2*	13[A], 14[A]
	Acetone	100 µL	*12*	*3*		14[A], 10[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF25CY.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF25CY
Date Plated: 4/5/2018
Evaluation Period: 4/9/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537		5000 µg	*7*	*1*	*0.9*	6[A], 8[A]
		1500 µg	*7*	*0*	*0.9*	7[A], 7[A]
		500 µg	*6*	*1*	*0.8*	7[A], 5[A]
		150 µg	*11*	*1*	*1.4*	11[A], 10[A]
		50.0 µg	*8*	*2*	*1.0*	9[A], 6[A]
		15.0 µg	*6*	*1*	*0.8*	7[A], 5[A]
		5.00 µg	*9*	*3*	*1.1*	11[A], 7[A]
		1.50 µg	*11*	*4*	*1.4*	8[A], 14[A]
	Acetone	100 µL	*8*	*1*		9[A], 7[A]
WP2uvrA		5000 µg	*27*	*1*	*1.3*	27[A], 26[A]
		1500 µg	*30*	*4*	*1.4*	27[A], 33[A]
		500 µg	*20*	*8*	*1.0*	26[A], 14[A]
		150 µg	*29*	*8*	*1.4*	23[A], 34[A]
		50.0 µg	*24*	*4*	*1.1*	27[A], 21[A]
		15.0 µg	*25*	*2*	*1.2*	23[A], 26[A]
		5.00 µg	*24*	*1*	*1.1*	24[A], 23[A]
		1.50 µg	*20*	*5*	*1.0*	23[A], 16[A]
	Acetone	100 µL	*21*	*3*		23[A], 19[A]
TA98	**2NF**	1.00 µg	*87*	*0*	*6.2*	87[A], 87[A]
TA100	**SA**	1.00 µg	*674*	*180*	*9.4*	801[A], 547[A]
TA1535	**SA**	1.00 µg	*506*	*10*	*42.2*	499[A], 513[A]
TA1537	**9AAD**	75.0 µg	*729*	*42*	*91.1*	759[A], 699[A]
WP2uvrA	**MMS**	1000 µg	*398*	*2*	*19.0*	396[A], 399[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF25CY.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF25CY
Date Plated: 4/5/2018
Evaluation Period: 4/9/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98		5000 µg	*21*	*3*	*1.0*	23[A], 19[A]
		1500 µg	*18*	*4*	*0.9*	15[A], 21[A]
		500 µg	*24*	*1*	*1.1*	23[A], 25[A]
		150 µg	*18*	*5*	*0.9*	14[A], 21[A]
		50.0 µg	*30*	*0*	*1.4*	30[A], 30[A]
		15.0 µg	*23*	*6*	*1.1*	27[A], 19[A]
		5.00 µg	*25*	*0*	*1.2*	25[A], 25[A]
		1.50 µg	*23*	*6*	*1.1*	18[A], 27[A]
	Acetone	100 µL	*21*	*2*		19[A], 22[A]
TA100		5000 µg	*104*	*7*	*1.3*	109[A], 99[A]
		1500 µg	*99*	*1*	*1.2*	100[A], 98[A]
		500 µg	*97*	*20*	*1.2*	111[A], 83[A]
		150 µg	*103*	*2*	*1.2*	104[A], 101[A]
		50.0 µg	*92*	*1*	*1.1*	91[A], 93[A]
		15.0 µg	*83*	*11*	*1.0*	90[A], 75[A]
		5.00 µg	*96*	*11*	*1.2*	103[A], 88[A]
		1.50 µg	*85*	*6*	*1.0*	89[A], 80[A]
	Acetone	100 µL	*83*	*1*		82[A], 84[A]
TA1535		5000 µg	*13*	*6*	*1.1*	9[A], 17[A]
		1500 µg	*14*	*1*	*1.2*	14[A], 13[A]
		500 µg	*17*	*1*	*1.4*	16[A], 17[A]
		150 µg	*12*	*2*	*1.0*	13[A], 10[A]
		50.0 µg	*9*	*4*	*0.8*	6[A], 11[A]
		15.0 µg	*12*	*4*	*1.0*	14[A], 9[A]
		5.00 µg	*13*	*0*	*1.1*	13[A], 13[A]
		1.50 µg	*13*	*3*	*1.1*	15[A], 11[A]
	Acetone	100 µL	*12*	*4*		14[A], 9[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF25CY.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF25CY
Date Plated: 4/5/2018
Evaluation Period: 4/9/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537		5000 µg	*11*	*4*	*0.9*	8[A], 14[A]
		1500 µg	*11*	*1*	*0.9*	11[A], 10[A]
		500 µg	*8*	*2*	*0.7*	9[A], 6[A]
		150 µg	*13*	*2*	*1.1*	11[A], 14[A]
		50.0 µg	*15*	*8*	*1.3*	21[A], 9[A]
		15.0 µg	*7*	*6*	*0.6*	3[A], 11[A]
		5.00 µg	*11*	*4*	*0.9*	8[A], 14[A]
		1.50 µg	*9*	*1*	*0.8*	8[A], 10[A]
	Acetone	100 µL	*12*	*1*		11[A], 13[A]
WP2uvrA		5000 µg	*34*	*1*	*1.1*	33[A], 35[A]
		1500 µg	*40*	*9*	*1.3*	46[A], 33[A]
		500 µg	*34*	*4*	*1.1*	31[A], 36[A]
		150 µg	*25*	*1*	*0.8*	25[A], 24[A]
		50.0 µg	*30*	*1*	*1.0*	31[A], 29[A]
		15.0 µg	*18*	*9*	*0.6*	24[A], 11[A]
		5.00 µg	*39*	*4*	*1.3*	41[A], 36[A]
		1.50 µg	*31*	*3*	*1.0*	33[A], 29[A]
	Acetone	100 µL	*31*	*6*		27[A], 35[A]
TA98	**2AA**	1.00 µg	*196*	*48*	*9.3*	162[A], 230[A]
TA100	**2AA**	2.00 µg	*866*	*29*	*10.4*	845[A], 886[A]
TA1535	**2AA**	1.00 µg	*102*	*9*	*8.5*	95[A], 108[A]
TA1537	**2AA**	2.00 µg	*94*	*20*	*7.8*	80[A], 108[A]
WP2uvrA	**2AA**	15.0 µg	*339*	*71*	*10.9*	288[A], 389[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF25CY.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF25CY
Date Plated: 4/20/2018
Evaluation Period: 4/23/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98		5000 µg	*24*	*6*	*1.3*	29[A], 25[A], 18[A]
		1500 µg	*15*	*4*	*0.8*	19[A], 15[A], 11[A]
		500 µg	*23*	*4*	*1.3*	19[A], 22[A], 27[A]
		150 µg	*21*	*2*	*1.2*	22[A], 18[A], 22[A]
		50.0 µg	*20*	*3*	*1.1*	23[A], 18[A], 19[A]
		15.0 µg	*22*	*4*	*1.2*	26[A], 21[A], 18[A]
	Acetone	100 µL	*18*	*3*		21[A], 17[A], 15[A]
TA100		5000 µg	*87*	*5*	*1.0*	83[A], 86[A], 93[A]
		1500 µg	*88*	*11*	*1.0*	83[A], 101[A], 81[A]
		500 µg	*82*	*11*	*1.0*	73[A], 95[A], 79[A]
		150 µg	*88*	*14*	*1.0*	91[A], 73[A], 101[A]
		50.0 µg	*87*	*7*	*1.0*	95[A], 86[A], 81[A]
		15.0 µg	*85*	*4*	*1.0*	89[A], 82[A], 83[A]
	Acetone	100 µL	*84*	*12*		72[A], 84[A], 96[A]
TA1535		5000 µg	*12*	*2*	*0.9*	13[A], 13[A], 10[A]
		1500 µg	*9*	*1*	*0.6*	8[A], 10[A], 10[A]
		500 µg	*13*	*3*	*0.9*	13[A], 16[A], 10[A]
		150 µg	*12*	*4*	*0.9*	7[A], 15[A], 13[A]
		50.0 µg	*16*	*4*	*1.1*	11[A], 19[A], 18[A]
		15.0 µg	*14*	*4*	*1.0*	9[A], 15[A], 17[A]
	Acetone	100 µL	*14*	*5*		19[A], 11[A], 11[A]
TA1537		5000 µg	*5*	*3*	*0.8*	5[A], 2[A], 8[A]
		1500 µg	*6*	*3*	*1.0*	9[A], 7[A], 3[A]
		500 µg	*5*	*1*	*0.8*	6[A], 5[A], 5[A]
		150 µg	*6*	*2*	*1.0*	6[A], 5[A], 8[A]
		50.0 µg	*4*	*3*	*0.7*	7[A], 1[A], 5[A]
		15.0 µg	*7*	*2*	*1.2*	9[A], 6[A], 6[A]
	Acetone	100 µL	*6*	*1*		7[A], 5[A], 5[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF25CY.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF25CY
Date Plated: 4/20/2018
Evaluation Period: 4/23/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA		5000 µg	*22*	*1*	*0.8*	22[A], 21[A], 22[A]
		1500 µg	*26*	*5*	*0.9*	29[A], 29[A], 21[A]
		500 µg	*24*	*3*	*0.9*	26[A], 21[A], 26[A]
		150 µg	*33*	*3*	*1.2*	35[A], 34[A], 29[A]
		50.0 µg	*37*	*5*	*1.3*	31[A], 39[A], 40[A]
		15.0 µg	*24*	*3*	*0.9*	23[A], 22[A], 27[A]
	Acetone	100 µL	*28*	*5*		22[A], 31[A], 31[A]
TA98	**2NF**	1.00 µg	*69*	*9*	*3.8*	59[A], 75[A], 74[A]
TA100	**SA**	1.00 µg	*616*	*9*	*7.3*	626[A], 611[A], 611[A]
TA1535	**SA**	1.00 µg	*508*	*13*	*36.3*	522[A], 505[A], 496[A]
TA1537	**9AAD**	75.0 µg	*752*	*214*	*125.3*	621[A], 999[A], 635[A]
WP2uvrA	**MMS**	1000 µg	*386*	*18*	*13.8*	404[A], 384[A], 369[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF25CY.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF25CY
Date Plated: 4/20/2018
Evaluation Period: 4/23/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98		5000 µg	*29*	*4*	*1.0*	26[A], 33[A], 27[A]
		1500 µg	*30*	*6*	*1.1*	35[A], 32[A], 23[A]
		500 µg	*25*	*5*	*0.9*	22[A], 23[A], 31[A]
		150 µg	*26*	*8*	*0.9*	35[A], 25[A], 19[A]
		50.0 µg	*30*	*12*	*1.1*	35[A], 39[A], 16[A]
		15.0 µg	*30*	*5*	*1.1*	25[A], 35[A], 29[A]
	Acetone	100 µL	*28*	*5*		25[A], 26[A], 34[A]
TA100		5000 µg	*106*	*3*	*1.0*	107[A], 108[A], 103[A]
		1500 µg	*97*	*9*	*0.9*	105[A], 87[A], 98[A]
		500 µg	*106*	*3*	*1.0*	106[A], 103[A], 108[A]
		150 µg	*110*	*10*	*1.1*	114[A], 99[A], 117[A]
		50.0 µg	*109*	*8*	*1.0*	114[A], 114[A], 100[A]
		15.0 µg	*94*	*4*	*0.9*	97[A], 95[A], 90[A]
	Acetone	100 µL	*104*	*4*		100[A], 106[A], 107[A]
TA1535		5000 µg	*10*	*1*	*0.6*	10[A], 11[A], 9[A]
		1500 µg	*12*	*1*	*0.7*	11[A], 13[A], 13[A]
		500 µg	*12*	*2*	*0.7*	14[A], 11[A], 11[A]
		150 µg	*13*	*4*	*0.8*	9[A], 14[A], 16[A]
		50.0 µg	*11*	*3*	*0.6*	9[A], 14[A], 11[A]
		15.0 µg	*13*	*5*	*0.8*	16[A], 8[A], 16[A]
	Acetone	100 µL	*17*	*1*		18[A], 18[A], 16[A]
TA1537		5000 µg	*7*	*1*	*0.8*	7[A], 6[A], 7[A]
		1500 µg	*8*	*3*	*0.9*	5[A], 10[A], 9[A]
		500 µg	*9*	*3*	*1.0*	11[A], 5[A], 11[A]
		150 µg	*8*	*3*	*0.9*	10[A], 10[A], 5[A]
		50.0 µg	*7*	*4*	*0.8*	10[A], 2[A], 9[A]
		15.0 µg	*9*	*2*	*1.0*	11[A], 7[A], 9[A]
	Acetone	100 µL	*9*	*1*		10[A], 9[A], 8[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4 (CONT.)
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF25CY.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF25CY
Date Plated: 4/20/2018
Evaluation Period: 4/23/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA		5000 µg	*33*	*8*	*1.0*	40[A], 24[A], 34[A]
		1500 µg	*31*	*2*	*0.9*	33[A], 29[A], 32[A]
		500 µg	*28*	*9*	*0.8*	30[A], 19[A], 36[A]
		150 µg	*32*	*10*	*0.9*	43[A], 23[A], 31[A]
		50.0 µg	*29*	*4*	*0.9*	33[A], 25[A], 29[A]
		15.0 µg	*28*	*8*	*0.8*	29[A], 19[A], 35[A]
	Acetone	100 µL	*34*	*4*		38[A], 31[A], 34[A]
TA98	**2AA**	1.00 µg	*241*	*21*	*8.6*	233[A], 225[A], 264[A]
TA100	**2AA**	2.00 µg	*837*	*47*	*8.0*	889[A], 824[A], 798[A]
TA1535	**2AA**	1.00 µg	*81*	*11*	*4.8*	91[A], 82[A], 70[A]
TA1537	**2AA**	2.00 µg	*44*	*5*	*4.9*	47[A], 47[A], 39[A]
WP2uvrA	**2AA**	15.0 µg	*419*	*41*	*12.3*	465[A], 407[A], 386[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values
2016
revertants per plate

Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	

SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control

14. APPENDIX II: Study Protocol and Amendment

PROTOCOL AMENDMENT 1

BioReliance Study No.: AF25CY.503.BTL; **Sponsor No.**

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 1500, 500, 150, 50.0 and 15.0 µg per plate.

   Reason: To specify the dose levels to be used for the confirmatory mutagenicity assay based on the toxicity and precipitate profiles observed in the initial toxicity-mutation assay.

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS
BioReliance Study Director

Date

BioReliance Study Management

14-APR-2018
Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF25CY.503.BTL

1. KEY PERSONNEL

Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	05-April-2018
Proposed Experimental Completion Date	04-May-2018
Proposed Report Date	18-May-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

NOTE: Follow sponsor's dispensing information when weighing compound

Characterization of Test substance
Characterization of the Test substance is the responsibility of the Sponsor.

Test substance Reserve Sample
A reserve sample of the Test substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test substance in Vehicle
Stability of Test substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test substance and Dose Formulations
All unused Test Article will be returned to the sponsor prior to report finalization, unless the test article is used on another study, using the following information.

Residual dose formulations will be discarded after use.

7. TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels

TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay

TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System

Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring

The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification

On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values

Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean vehicle control value (this reduction must be

accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains
- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)
- **Japanese (METI) Tables**
- **IUCLID Report**

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

Study Director and Test Facility Management Approvals

Emily Dakoulas, BS
BioReliance Study Director

03 APR 2018
Date

BioReliance Study Management

03 Apr 2018
Date

15. APPENDIX III: Certificate of Analysis

16. APPENDIX IV: Japanese ISHL Tables

Report of Results of Bacterial Reverse Mutation Study

1. General Items

Name of the new chemical substance (IUPAC nomenclature)	-		
Other name (CA Index Name)			
Structural formula or rational formula (or outline of manufacturing method, in case both are unknown)	-		
Purity of the new chemical substance tested	99.09%	Lot No. of the new chemical substance tested	12639
Name and concentration of impurities	-		
CAS No.		Vapor pressure	No data available
Molecular weight		Partition coefficient	No data available
Melting point	No data available	Appearance at ordinary temperature	Clear colorless liquid
Initial Boiling point and boiling point range	73ºC		
Stability	The stability to define the test substance has not been determined.		

Degree of solubility in solvent	Solvent	Solubility	Stability of the test substance in the solvent
	Water	Not available	Not available
	DMSO	Insoluble	Not available
	Acetone	The test substance formed a clear solution in acetone at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance	Not available
	Ethanol	Insoluble	Not available
Remarks: -			

2. Tester Strains

Name of tester strains	Obtained from	Date obtained
TA100	*Salmonella* tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.	16 January 2004
TA1535		13 November 2002
TA98		
TA1537		
WP2*uvr*A		1 July 1987

3. S9 mix

(1) Source of S9 (Encircle the applicable number, and fill in the columns)

Source	1. Made in-house	2. Purchase (supplier: Molecular Toxicology, Inc., Boone, NC, USA)
Date of production	31 October 2017	
Lot No. (in case of purchase)	3880	
Storage temperature	-60ºC or colder	

(2) Preparation of S9

Animal used		Inducing substance	
Species, Strain	Rat, Sprague-Dawley	Name	Aroclor 1254
Sex	Male	Administration method	Intraperitoneal injection
Age (in weeks)	5 to 6	Administration period and amount (g/Kg-b.w.)	Single injection of 500 mg/kg 5 days before sacrifice
Body weight	175 to 199 g		

(3) Composition of S9 Mix

Constituents	Amount in 1 ml of S9 Mix	Constituents	Amount in 1 ml S9 Mix
S9	0.1 ml	NADPH	Not applicable
$MgCl_2$	0.008 mmol	NADH	Not applicable
KCl	0.033 mmol	Na-phosphate buffer	0.100 mmol
Glucose-6-phosphate	0.005 mmol	Others : NADP Water	0.004 mmol balance

4. Preparation of Test Substance Solution

Solvent used	Name	Supplier	Lot No	Grade and/or Purity(%)
	Acetone	Sigma-Aldrich	SHBJ2689	99.72%
Reason for selection of the solvent	Acetone was selected as the solvent of choice based on the solubility of the test substance and compatibility with the target cells.			
Appearance of the test substance solution	The test substance formed a clear solution in acetone at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.			
Method of suspending when the test substance is hardly soluble	-			
Storage time and temperature of the test substance solution from preparation until use	< 2 hours at ambient temperature Dilutions were prepared immediately before use and were delivered to the test system at room temperature under filtered light.			
Conversion for the purity	Nil			

5. Conditions of Pre-culture

(1) Conditions

Nutrient broth	Name	Supplier	Lot No.
	Oxoid Nutrient Broth No. 2	Oxoid Ltd.	2202237
Time of pre-culture	Approximately 12 hours		
Culture vessel (shape and volume)	shape: cylinder, 150 mL		
Volume of culture medium	30 to 50 mL	Amount of strain inoculated	1 colony or ~100 µl

(2) Cell viability at the end of pre-culture

Name of tester strains		Base-pair change type			Frame-shift type	
		TA100	TA1535	WP2*uvr*A	TA98	TA1537
Cell numbers ($\times 10^9$/ml)	Initial Toxicity-Mutation Assay	1.0	1.6	2.6	1.4	1.7
	Confirmatory Mutagenicity Assay	1.1	1.2	3.2	1.8	1.4
Counting method		1. Monitor by O.D. value 2. Verify by stepwise dilution method				

6. Minimum glucose agar plate medium (Encircle the applicable number, and fill in columns)

Source	1. Made-in house 2. Purchase (supplier: Molecular Toxicology, Inc., Boone, NC, USA)
Prepared on	13 March 2018 (Lot No. 50327), 28 March 2018 (Lot Nos. 50413 and 50414)
Lot No. (in case of purchase)	50327, 50413, 50414
Name, manufacturer of the used agar	Becton Dickinson, BBL Select Lots 6315897 (Lot No. 50327), 7142520 (Lot Nos. 50413 and 50414)

7. Test Method (Encircle the applicable number, and fill in columns)

(1) Test method and reason for selection

Test method	1. Pre-incubation method 2. Plate method 3. Others ( )
Reason for selection	Sponsor's request

(2) Conditions

Composition	Bacterial suspension	100 µl
	Test substance solution	100 µl
	Phosphate buffer (in case of direct method)	0.5 ml
	S9 mix (in case of metabolic activation method)	0.5 ml
	Top agar solution	2.0 ml
	Others (minimal bottom agar)	25 ml
Pre-incubation	Temperature	ºC
	Time	min
Incubation	Temperature	37±2 ºC
	Time	48 to 72 hours

8. Colony Counting Method

Colony Counting Method	1. Manual counting 2. Automatic counting
Correction	1. No 2. Yes

9. Test Results

Study Name: AF25CY.503.BTL
Experiment: B1
Assay Conditions: Plate incorporation assay

Study Code: AF25CY
Date Plated: 4/5/2018
Date Counted: 4/9/2018

	Dose Level Per Plate	Number of Revertants, (Mean), +/- SD											
		Base-pair Substitution Strains							Frameshift Strains				
		TA100		**TA1535**		**WP2uvrA**			**TA98**		**TA1537**		
S9-Mix (-)	Solvent Control (Acetone)	64 80	(72) 11	14 10	(12) 3	23 19	(21) 3		11 17	(14) 4	9 7	(8) 1
	5000 µg	103 83	(93) 14	16 14	(15) 1	27 26	(27) 1		11 19	(15) 6	6 8	(7) 1
	1500 µg	86 90	(88) 3	18 17	(18) 1	27 33	(30) 4		15 14	(15) 1	7 7	(7) 0
	500 µg	79 92	(86) 9	15 17	(16) 1	26 14	(20) 8		15 14	(15) 1	7 5	(6) 1
	150 µg	105 105	(105) 0	8 11	(10) 2	23 34	(29) 8		17 24	(21) 5	11 10	(11) 1
	50.0 µg	108 59	(84) 35	14 19	(17) 4	27 21	(24) 4		16 16	(16) 0	9 6	(8) 2
	15.0 µg	74 83	(79) 6	10 8	(9) 1	23 26	(25) 2		15 14	(15) 1	7 5	(6) 1
	5.00 µg	82 99	(91) 12	11 9	(10) 1	24 23	(24) 1		17 26	(22) 6	11 7	(9) 3
	1.50 µg	81 86	(84) 4	13 14	(14) 1	23 16	(20) 5		7 15	(11) 6	8 14	(11) 4

	Name	SA		SA		MMS			2NF		9AAD	
	Dose	1.00 µg		1.00 µg		1000 µg			1.00 µg		75.0 µg	
S9-Mix (-)	Level No. of Revertants	801 547	(674) 180	499 513	(506) 10	396 399	(398) 2		87 87	(87) 0	759 699	(729) 42

Key to Positive Controls

SA	sodium azide
MMS	methyl methanesulfonate
2NF	2-nitrofluorene
9AAD	9-Aminoacridine

[Note]

1. If bacterial cell growth suppression is confirmed (toxicity observed), mark the relevant figure with an asterisk (*) in the top right corner.
2. Record the average number of colonies for each plate in the parentheses.
3. If a sediment forms on the plate, mark that dosage with a †.
4. Record the names of positive substances shown in abbreviated form in the margin.

Study Name: AF25CY.503.BTL
Experiment: B1
Assay Conditions: Plate incorporation assay

Study Code: AF25CY
Date Plated: 4/5/2018
Date Counted: 4/9/2018

	Dose Level Per Plate	Number of Revertants, (Mean), +/- SD											
		Base-pair Substitution Strains							Frameshift Strains				
		TA100		**TA1535**		**WP2uvrA**			**TA98**		**TA1537**		
S9-Mix (+)	Solvent Control (Acetone)	82 84	(83) 1	14 9	(12) 4	27 35	(31) 6		19 22	(21) 2	11 13	(12) 1	
	5000 µg	109 99	(104) 7	9 17	(13) 6	33 35	(34) 1		23 19	(21) 3	8 14	(11) 4	
	1500 µg	100 98	(99) 1	14 13	(14) 1	46 33	(40) 9		15 21	(18) 4	11 10	(11) 1	
	500 µg	111 83	(97) 20	16 17	(17) 1	31 36	(34) 4		23 25	(24) 1	9 6	(8) 2	
	150 µg	104 101	(103) 2	13 10	(12) 2	25 24	(25) 1		14 21	(18) 5	11 14	(13) 2	
	50.0 µg	91 93	(92) 1	6 11	(9) 4	31 29	(30) 1		30 30	(30) 0	21 9	(15) 8	
	15.0 µg	90 75	(83) 11	14 9	(12) 4	24 11	(18) 9		27 19	(23) 6	3 11	(7) 6	
	5.00 µg	103 88	(96) 11	13 13	(13) 0	41 36	(39) 4		25 25	(25) 0	8 14	(11) 4	
	1.50 µg	89 80	(85) 6	15 11	(13) 3	33 29	(31) 3		18 27	(23) 6	8 10	(9) 1	

	Name	2AA		2AA		2AA			2AA		2AA		
S9-Mix (+)	Dose Level	2.00 µg		1.00 µg		15.0 µg			1.00 µg		2.00 µg		
	No. of Revertants	845 886	(866) 29	95 108	(102) 9	288 389	(339) 71		162 230	(196) 48	80 108	(94) 20	

Key to Positive Controls

2AA	2-aminoanthracene

[Note]

1. If bacterial cell growth suppression is confirmed (toxicity observed), mark the relevant figure with an asterisk (*) in the top right corner.
2. Record the average number of colonies for each plate in the parentheses.
3. If a sediment forms on the plate, mark that dosage with a †.
4. Record the names of positive substances shown in abbreviated form in the margin.

Study Name: AF25CY.503.BTL
Experiment: B2
Assay Conditions: Plate incorporation assay

Study Code: AF25CY
Date Plated: 4/20/2018
Date Counted: 4/23/2018

	Dose Level Per Plate	Number of Revertants, (Mean), +/- SD											
		Base-pair Substitution Strains							Frameshift Strains				
		TA100		**TA1535**		**WP2uvrA**			**TA98**		**TA1537**		
S9-Mix (-)	Solvent Control (Acetone)	72	(84)	19	(14)	22	(28)		21	(18)	7	(6)	
		84	12	11	5	31	5		17	3	5	1	
		96		11		31			15		5		
	5000 µg	83	(87)	13	(12)	22	(22)		29	(24)	5	(5)	
		86	5	13	2	21	1		25	6	2	3	
		93		10		22			18		8		
	1500 µg	83	(88)	8	(9)	29	(26)		19	(15)	9	(6)	
		101	11	10	1	29	5		15	4	7	3	
		81		10		21			11		3		
	500 µg	73	(82)	13	(13)	26	(24)		19	(23)	6	(5)	
		95	11	16	3	21	3		22	4	5	1	
		79		10		26			27		5		
	150 µg	91	(88)	7	(12)	35	(33)		22	(21)	6	(6)	
		73	14	15	4	34	3		18	2	5	2	
		101		13		29			22		8		
	50.0 µg	95	(87)	11	(16)	31	(37)		23	(20)	7	(4)	
		86	7	19	4	39	5		18	3	1	3	
		81		18		40			19		5		
	15.0 µg	89	(85)	9	(14)	23	(24)		26	(22)	9	(7)	
		82	4	15	4	22	3		21	4	6	2	
		83		17		27			18		6		

	Name	SA		SA		MMS			2NF		9AAD	
S9-Mix (-)	Dose Level	1.00 µg		1.00 µg		1000 µg			1.00 µg		75.0 µg	
	No. of Revertants	626	(616)	522	(508)	404	(386)		59	(69)	621	(752)
		611	9	505	13	384	18		75	9	999	214
		611		496		369			74		635	

Key to Positive Controls

SA	sodium azide
MMS	methyl methanesulfonate
2NF	2-nitrofluorene
9AAD	9-Aminoacridine

[Note]

1. If bacterial cell growth suppression is confirmed (toxicity observed), mark the relevant figure with an asterisk (*) in the top right corner.
2. Record the average number of colonies for each plate in the parentheses.
3. If a sediment forms on the plate, mark that dosage with a †.
4. Record the names of positive substances shown in abbreviated form in the margin.

Study Name: AF25CY.503.BTL
Experiment: B2
Assay Conditions: Plate incorporation assay

Study Code: AF25CY
Date Plated: 4/20/2018
Date Counted: 4/23/2018

	Dose Level Per Plate	Number of Revertants, (Mean), +/- SD									
		Base-pair Substitution Strains							Frameshift Strains		
		TA100		**TA1535**		**WP2uvrA**			**TA98**	**TA1537**	
S9-Mix (+)	Solvent Control (Acetone)	100	(104)	18	(17)	38	(34)		25 (28)	10	(9)
		106	4	18	1	31	4		26 5	9	1
		107		16		34			34	8	
	5000 µg	107	(106)	10	(10)	40	(33)		26 (29)	7	(7)
		108	3	11	1	24	8		33 4	6	1
		103		9		34			27	7	
	1500 µg	105	(97)	11	(12)	33	(31)		35 (30)	5	(8)
		87	9	13	1	29	2		32 6	10	3
		98		13		32			23	9	
	500 µg	106	(106)	14	(12)	30	(28)		22 (25)	11	(9)
		10	3	11	2	19	9		23 5	5	3
		108		11		36			31	11	
	150 µg	114	(110)	9	(13)	43	(32)		35 (26)	10	(8)
		99	10	14	4	23	10		25 8	10	3
		117		16		31			19	5	
	50.0 µg	114	(109)	9	(11)	33	(29)		35 (30)	10	(7)
		114	8	14	3	25	4		39 12	2	4
		100		11		29			16	9	
	15.0 µg	97	(94)	16	(13)	29	(28)		25 (30)	11	(9)
		95	4	8	5	19	8		35 5	7	2
		90		16		35			29	9	

	Name	2AA		2AA		2AA			2AA	2AA	
S9-Mix (+)	Dose	2.00 µg		1.00 µg		15.0 µg			1.00 µg	2.00 µg	
	Level No. of Revertants	889	(837)	91	(81)	465	(419)		233 (241)	47	(44)
		824	47	82	11	407	41		225 21	47	5
		798		70		386			264	39	

Key to Positive Controls

2AA	2-aminoanthracene

[Note]

1. If bacterial cell growth suppression is confirmed (toxicity observed), mark the relevant figure with an asterisk (*) in the top right corner.
2. Record the average number of colonies for each plate in the parentheses.
3. If a sediment forms on the plate, mark that dosage with a †.
4. Record the names of positive substances shown in abbreviated form in the margin.

(2) Judgment of the results

Judgment (Encircle one)	Negative	Positive
All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9		

(3) Referential matters

The vehicle and positive control values indicate that all tester strains were functioning correctly and were capable of detecting a mutagen.

10. Others

Testing facility	Name	BioReliance Corporation
	Address	9630 Medical Center Drive Rockville, MD 20850, USA
Study director	Name/Title	Emily Dakoulas, BS
	Years of experience	~ 20 years
Study No.	AF25CY.503.BTL	
Test dates *	Initial day: 03 April 2018 Final day: 21 June 2018	

* Initial day of the test: The date of Study Director's signature on the protocol
Final day of the test: The date of final report issue

11. Specific Activity (In the case of positive results)

	Bacterial tester strains	-S9 mix		+ S9 mix	
		*1) Specific activity	*2) Concentration (μg/plate)	*1) Specific activity	*2) Concentration (μg/plate)
Initial Toxicity-Mutation Assay	Not applicable (negative)				
Confirmatory Mutagenicity Assay					

*1) Number of revertant colonies per 1 mg test substance
*2) The concentration of the maximum specific activity was entered.

Appendix List of Positive Control Reagents

Reagent Name	Abbrev.	Supplier	Lot No.	Purity (%)
Methyl methanesulfonate	MMS	Sigma-Aldrich Chemical Co., Inc.	MKBX5165V	99.5
9-aminoacridine	9AAD	Sigma-Aldrich Chemical Co., Inc.	BCBK1177V	99.5
Sodium azide	SA	Sigma-Aldrich Chemical Co., Inc.	MKBT8080V	99.8
2-aminoanthracene	2AA	Sigma-Aldrich Chemical Co., Inc.	STBD3302V	97.5
2-nitrofluorene	2NF	Sigma-Aldrich Chemical Co., Inc.	S43858V	99.4

17. APPENDIX V: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test for Induction of: Reverse mutation in bacterial cells

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

Metabolizing System: Aroclor-induced rat liver S9

Vehicle for Test Substance: Acetone

Treatment: Plate incorporation

Cytotoxic Effects: None

Genotoxic Effects: None

No. of Independent Assays: 2

No. of Replicate Cultures: 2 (B1) and 3 (B2)

Test Substance:

Study No.: AF25CY.503.BTL

No. Cells Analyzed/Culture: 1.0 to 3.2 x 10^8 cells per plate

GLP Compliance: Yes

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Date(s) of Treatment: 05 April 2018 (B1) and 20 April 2018 (B2)

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B1: Initial Toxicity Mutation Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Acetone	100 µL/plate	14 ± 4	72 ± 11	12 ± 3	8 ± 1	21 ± 3
		1.50	11 ± 6	84 ± 4	14 ± 1	11 ± 4	20 ± 5
		5.00	22 ± 6	91 ± 12	10 ± 1	9 ± 3	24 ± 1
		15.0	15 ± 1	79 ± 6	9 ± 1	6 ± 1	25 ± 2
		50.0	16 ± 0	84 ± 35	17 ± 4	8 ± 2	24 ± 4
		150	21 ± 5	105 ± 0	10 ± 2	11 ± 1	29 ± 8
		500	15 ± 1	86 ± 9	16 ± 1	6 ± 1	20 ± 8
		1500	15 ± 1	88 ± 3	18 ± 1	7 ± 0	30 ± 4
		5000	15 ± 6	93 ± 14	15 ± 1	7 ± 1	27 ± 1
	2NF	1.00	87 ± 0				
	SA	1.00		674 ± 180	506 ± 10		
	9AAD	75.0				729 ± 42	
	MMS	1000					398 ± 2
With Activation	Acetone	100 µL/plate	21 ± 2	83 ± 1	12 ± 4	12 ± 1	31 ± 6
		1.50	23 ± 6	85 ± 6	13 ± 3	9 ± 1	31 ± 3
		5.00	25 ± 0	96 ± 11	13 ± 0	11 ± 4	39 ± 4
		15.0	23 ± 6	83 ± 11	12 ± 4	7 ± 6	18 ± 9
		50.0	30 ± 0	92 ± 1	9 ± 4	15 ± 8	30 ± 1
		150	18 ± 5	103 ± 2	12 ± 2	13 ± 2	25 ± 1
		500	24 ± 1	97 ± 20	17 ± 1	8 ± 2	34 ± 4
		1500	18 ± 4	99 ± 1	14 ± 1	11 ± 1	40 ± 9
		5000	21 ± 3	104 ± 7	13 ± 6	11 ± 4	34 ± 1
	2AA	1.00	196 ± 48		102 ± 9		
	2AA	2.00		866 ± 29		94 ± 20	
	2AA	15.0					339 ± 71

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B2: Confirmatory Mutagenicity Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Acetone	100 µL/plate	18 ± 3	84 ± 12	14 ± 5	6 ± 1	28 ± 5
		15.0	22 ± 4	85 ± 4	14 ± 4	7 ± 2	24 ± 3
		50.0	20 ± 3	87 ± 7	16 ± 4	4 ± 3	37 ± 5
		150	21 ± 2	88 ± 14	12 ± 4	6 ± 2	33 ± 3
		500	23 ± 4	82 ± 11	13 ± 3	5 ± 1	24 ± 3
		1500	15 ± 4	88 ± 11	9 ± 1	6 ± 3	26 ± 5
		5000	24 ± 6	87 ± 5	12 ± 2	5 ± 3	22 ± 1
	2NF	1.00	69 ± 9				
	SA	1.00		616 ± 9	508 ± 13		
	9AAD	75.0				752 ± 214	
	MMS	1000					386 ± 18
With Activation	Acetone	100 µL/plate	28 ± 5	104 ± 4	17 ± 1	9 ± 1	34 ± 4
		15.0	30 ± 5	94 ± 4	13 ± 5	9 ± 2	28 ± 8
		50.0	30 ± 12	109 ± 8	11 ± 3	7 ± 4	29 ± 4
		150	26 ± 8	110 ± 10	13 ± 4	8 ± 3	32 ± 10
		500	25 ± 5	106 ± 3	12 ± 2	9 ± 3	28 ± 9
		1500	30 ± 6	97 ± 9	12 ± 1	8 ± 3	31 ± 2
		5000	29 ± 4	106 ± 3	10 ± 1	7 ± 1	33 ± 8
	2AA	1.00	241 ± 21		81 ± 11		
	2AA	2.00		837 ± 47		44 ± 5	
	2AA	15.0					419 ± 41

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

FINAL REPORT

Study Title

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH OPTIONAL HISTOLOGY

Test Article

(CAS

Test Guideline

OECD Guideline 437 (2013)

Authors

Allison Hilberer, M.S., DABT
Timothy Herpel, B.S.

Study Completion Date

2 August 2018

Performing Laboratory

Institute for In Vitro Sciences, Inc.
30 W. Watkins Mill Road, Suite 100
Gaithersburg, MD 20878

Study Number

18AD37.350049

Laboratory Project Number

9780

Sponsor Study Number

TABLE OF CONTENTS

STATEMENT OF COMPLIANCE

The Bovine Corneal Opacity And Permeability Assay with Optional Histology of the test article, (CAS was conducted in compliance with the U.S. EPA GLP Standards 40 CFR 792 in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the negative control have not been determined by the testing facility. However, the manufacturers (controls) provided Certificates of Analysis that are included in Appendix C.

The stability of the test article and negative control under the storage conditions at the testing facility and under the actual test conditions has not been determined by the testing facility and is not included in the final report.

Allison Hilberer, M.S., DABT
Study Director

2 August 2018
Date

QUALITY ASSURANCE STATEMENT

Study Title: Bovine Corneal Opacity and Permeability Assay with Optional Histology

Study Number: 18AD37.350049

Study Director: Allison Hilberer, M.S., DABT

A random sampling approach was used to select at least one in-process, laboratory phase to inspect for this study. The Quality Assurance Unit inspections specific to this study are listed in the table below. Procedures, documentation, equipment records, etc., were examined in order to assure that the study was performed in accordance with the the U.S. EPA GLP Standards 40 CFR 792 and to assure that the study was conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director and Management
Protocol and Initial Paperwork	30-Apr-18	30-Apr-18
Addition of Fluorescein Solution	03-May-18	03-May-18
Final Report and Data	30-July-18	31-July-18

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Amanda K. Ulrey, RQAP-GLP
Quality Assurance

02-Aug-2018
Date

SIGNATURE PAGE

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH OPTIONAL HISTOLOGY

Initiation Date:	26 April 2018
Completion Date:	2 August 2018
Testing Facility:	Institute for In Vitro Sciences, Inc. 30 W. Watkins Mill Road, Suite 100 Gaithersburg, MD 20878
Archive Location:	Institute for In Vitro Sciences, Inc. Gaithersburg, MD 20878
Director of Laboratory Operations:	Greg Mun, B.A.

Study Director:  2 August 2018

Allison Hilberer, M.S., DABT Date

TEST ARTICLE RECEIPT

IIVS Test Article Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions*
18AD37	(CAS	clear colorless non-viscous liquid	6 April 2018	room temperature

* - Protected from exposure to light

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH OPTIONAL HISTOLOGY

INTRODUCTION

The Bovine Corneal Opacity and Permeability Assay (BCOP) was used to assess the potential ocular irritancy of the test article to isolated bovine corneas. Bovine corneas, obtained as a by-product from freshly slaughtered animals, were mounted in special holders and exposed to the test article. An *In Vitro* Score was determined for the test article based on the induction of opacity and permeability (to fluorescein) in the isolated bovine corneas. This study was conducted according to the OECD guideline 437, "*Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage*", adopted 26 July 2013[1].

The purpose of this study was to evaluate the potential ocular irritancy of the test article, supplied by , as measured by changes in opacity and permeability (to fluorescein) in isolated bovine corneas. Five corneas were treated with the test article. The laboratory phase of this study was conducted on 3 May 2018 at the Institute for In Vitro Sciences, Inc.

[1] OECD (2013),*Test No. 437: Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing

MATERIALS AND METHODS

Bovine Eyes

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals (J.W. TREUTH & SONS, Inc., Baltimore, MD). The eyes were excised and then placed in Hanks' Balanced Salt Solution, containing penicillin/streptomycin (HBSS), and transported to the laboratory on ice packs. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas was initiated.

Preparation of Corneas

The eyes were grossly examined for damage and those exhibiting defects were discarded. The tissue surrounding the eyeball was carefully pulled away and the cornea was excised such that a 2 to 3 mm rim of sclera was present around the cornea. The isolated corneas were then stored in a petri dish containing HBSS until they were mounted in a corneal holder. The corneas were mounted in the holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber was then positioned on top of the cornea and the screws were tightened. Starting with the posterior chamber, the two chambers were then filled with Minimum Essential Medium without phenol red, containing 1% fetal bovine serum and 2 mM L-glutamine (Complete MEM (without phenol red)). Each corneal holder was uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The corneal holders were incubated at 32 ± 1ºC for a minimum of 1 hour.

Controls

The positive control used in this study was ethanol (Pharmco). The negative control used in this study was sterile, deionized water (Quality Biological).

Test Article Preparation

The test article was received into IIVS in a stainless steel Hoke cylinder with 10 psig N_2 fitted with capped needle valves on both ends of the cylinder. The Sponsor provided instructions on removal of the test article from the vessel. On the day of testing, a small hose barb fitting was placed over one of the valves and an aliquot of the test article was placed into a labeled glass receiving vessel. The glass vessel was capped until dosing was initiated.

The test article was administered to the test system without dilution (neat).

Test Article pH Determination

The pH of the test article was determined using pH paper (EMD Millipore Corporation). The test article was added to 0-14 pH paper with 1.0 pH unit increments to approximate a narrow pH range. Next, the test article was added to 0-6 paper with 0.5 pH unit increments. The pH value obtained from the narrower range pH paper is presented in Table 1.

Bovine Corneal Opacity and Permeability Assay

After a minimum of 1 hour of incubation, the corneas were removed from the incubator. The medium was removed from both chambers and replaced with fresh Complete MEM (without

phenol red). The initial opacity was determined for each cornea using an Electro Designs OP-KIT opacitometer. The treatment of each cornea was identified with the test article number written in permanent marker on colored tape, affixed to each holder. The medium was then removed from the anterior chamber and replaced with the test article, positive control, or negative control.

Method for Testing Liquid or Surfactant Materials

The liquid test article, (CAS was tested neat. An aliquot of 750 µL of the test article, positive control, or negative control was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. Three corneas were incubated in the presence of the positive control at 32 ± 1ºC for 10 minutes. Three corneas were incubated in the presence of the negative control at 32 ± 1ºC for 10 minutes. Five corneas were incubated in the presence of the test article at 32 ± 1ºC for 10 minutes. After the 10-minute exposure time, the control or test article treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the control or test articles. The corneas were then given a final rinse with Complete MEM (without phenol red). The anterior chambers were refilled with fresh Complete MEM (without phenol red) and an opacity measurement was performed. The corneas were returned to the incubator for approximately 2 hours after which a final measure of opacity was obtained.

After the final opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was filled with fresh Complete MEM (without phenol red) and 1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at 32 ± 1ºC. At the end of the 90-minute incubation period, the medium was removed from the posterior chamber and placed into tubes numbered corresponding to chamber number. Aliquots of 360 µL from the numbered tubes were placed into their designated wells on a 96-well plate. The optical density at 490 nm (OD_{490}) was determined using a Molecular Devices *V*max kinetic microplate reader.

Fixation of Corneas

After the medium was removed for the permeability determination, each cornea was carefully separated from its corneal holder and transferred to an individual labeled tissue cassette containing a biopsy sponge. The endothelial surface of each cornea was placed on the sponge to protect it. The cassettes were placed in 10% neutral buffered formalin to fix the corneal tissue for at least 24 hours. The fixed corneas will be stored up to one year.

Histological Evaluation

As instructed by the Sponsor, a histological evaluation was not performed.

Presentation of Data

Opacity Measurement: The change in opacity for each cornea (including the negative control corneas) was calculated by subtracting the initial opacity reading from the final opacity reading. These values were then corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value of each treatment group was calculated by averaging the corrected opacity values of each cornea for that treatment condition.

Permeability Measurement: The mean OD_{490} value for the blank wells was calculated. The mean blank OD_{490} value was then subtracted from the raw OD_{490} value of each well (corrected OD_{490}). The final corrected OD_{490} values of the test article and the positive control were then calculated by subtracting the average corrected OD_{490} value of the negative control corneas from the corrected OD_{490} value of each treated cornea:

Final Corrected OD_{490} = (raw OD_{490} – mean blank OD_{490}) – average corrected negative control OD_{490}

The mean OD_{490} value of each treatment group was calculated by averaging the final corrected OD_{490} values of the treated corneas for that treatment condition.

The following formula was used to determine the *In Vitro* Score:

In Vitro Score = Mean Opacity Value + (15 x Mean OD_{490} Value)

Criteria for Determination of a Valid Test

The BCOP assay was accepted when the positive control (ethanol) produced an *In Vitro* Score that fell within two standard deviations of the historical mean.

RESULTS AND DISCUSSION

Bovine Corneal Opacity and Permeability Assay

Table 1 summarizes the opacity, permeability, and *In Vitro* Score for the test article and the positive control. Since the results of the positive control fell within two standard deviations of the historical mean (within a range of 38.7 to 63.5), the assay was considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

For regulatory purposes, the *In Vitro* Score (IVIS) cut-off values for identifying test chemicals as inducing serious eye damage (UN GHS Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) are found in the table below (OECD 437, adopted 26 July 2013). This guidance on categorization applies only to test articles evaluated using the appropriate standard protocols as described in OECD 437.

IVIS	UN GHS
≤3	No Category
>3; ≤55	No prediction can be made
>55	Category 1

For non-regulatory purposes, the following classification system was established by Sina et al.[2] based on studies with a wide range of test materials. While this classification system provides a good initial guide to interpretation of these *in vitro* data, these specific ranges may not be applicable to all classes of materials or other exposure times. Whenever possible, results should be compared to "benchmark" materials tested under similar exposure conditions.

In Vitro Score:

≤ 25	= mild irritant
from 25.1 to 55	= moderate irritant
from 55.1 and above	= severe irritant

[2] Sina, J.F., Galer, D.M., Sussman, R.G., Gautheron, P.D., Sargent, E.V., Leong, B., Shah, P.V., Curren, R.D., and Miller, K. (1995) A collaborative evaluation of seven alternatives to the Draize eye irritation test using pharmaceutical intermediates. **Fundamental and Applied Toxicology** 26:20-31.

Table 1
BCOP Results of the Test Article and the Positive Control

Assay Date	IIVS Test Article Number	Sponsor's Designation	Conc.	Exposure Time	Opacity Value	OD_{490} Value	*In Vitro* Score	pH
3 May 2018	18AD37	(CAS	Neat	10 Minutes	0.8	0.012	1.0	4.5
	Positive Control	Ethanol	Neat	10 Minutes	28.7	0.959	43.0	NA

NA – Not Applicable

Deviation

A SOP deviation occurred during the conduct of this study. The fluorescein 4 mg/mL stock solution used for the study was prepared on 16 April 2018 and stored at 2-8ºC (6 month shelf-life after preparation). On 2 May 2018, IIVS experienced an overnight power outage and all equipment was without power for approximately 7 hours. When power was restored, the refrigerator where the stock fluorescein was stored was outside the 2-8ºC range (9-10ºC). This deviation had no impact on the study results- the negative control corneas and positive control corneas produced optical density values as expected (mean optical density values of 0.007 and 0.959, respectively). In addition, 1:1000 dilution of fluorescein is prepared and read on the plate reader as R&D for every study and the results were as expected (optical density range of approximately 0.7 to 0.9); and further, upon preparation of a new batch of stock fluorescein on 21 June 2018, the new 4 mg/mL batch 1:1000 dilution and the study (affected) batch 1:1000 results were similar.

APPENDIX A

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH OPTIONAL HISTOLOGY

1.0 PURPOSE

The purpose of this study is to evaluate the potential ocular irritancy/toxicity of a test article as measured by the test article's ability to induce opacity and permeability to fluorescein in an isolated bovine cornea.

2.0 SPONSOR

3.0 IDENTIFICATION OF TEST ARTICLE(S) AND ASSAY CONTROLS

3.1 Test Article(s): See Protocol Attachment 1

3.2 Assay Controls: Positive: Ethanol (CAS #64-17-5) Neat (liquid test articles)
Imidazole (CAS #288-32-4) 20% (w/v) in Complete MEM (solid test articles)

Negative: Sterile deionized water or appropriate solvent

3.3 Determination of Strength, Purity, etc.

3.3.1 For GLP studies, the Institute for In Vitro Sciences, Inc. (IIVS) will attempt to secure documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions from the Sponsor. If the Sponsor is unable to provide such information, the final report will be generated with an exception noted in the Statement of Compliance.

3.3.2 IIVS will be responsible for the documentation of the analytical purity and composition of the positive and negative controls used. This may be accomplished by maintaining a certificate of analysis from the supplier.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Institute for In Vitro Sciences, Inc.

4.2 Address: 30 W. Watkins Mill Road, Suite 100
Gaithersburg, MD 20878

4.3 Study Director: Allison Hilberer, M.S., DABT

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 26 April 2018

5.2 Proposed Experimental Completion Date: 11 May 2018

5.3 Proposed Report Date: 6 July 2018

6.0 TEST SYSTEM

The test system (target tissue) is the isolated bovine cornea obtained as a by-product from freshly slaughtered animals. The procedures for preparing and handling the test system were developed by Gautheron et al. (1992). The assay measures two important components which are predictive of eye irritation; corneal opacity and permeability. Each cornea holder will be uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The treatment of each cornea will be identified with the test article number (or control) written in permanent marker on colored tape, affixed to each holder. Furthermore, when requested by the Sponsor, the depth and degree of injury may be assessed by histological evaluation.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The methods for the Bovine Corneal Opacity and Permeability Assay presented herein are based on the procedures described in the OECD test guideline "*Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage*" (OECD TG 437, adopted 26 July 2013).

Liquid test articles will be tested neat unless otherwise directed by the Sponsor. If the liquid test article is to be diluted, the study may begin with a solubility or miscibility test in sterile, deionized water or appropriate solvent designated by the Sponsor. Solid test articles will be diluted in sterile deionized water unless otherwise directed by the Sponsor. The pH of each neat (liquid) test article or diluted test article will be determined, if possible, and recorded. Three corneas treated with sterile, deionized water will serve as the negative control. Three corneas will be exposed to the positive control. Four or five corneas will be treated with each neat test article or test article solution/suspension.

One of two treatment methods will be used depending on the physical state and chemical characteristics (liquid or surfactant versus non-surfactant solid) of the test article. Changes in opacity and permeability to fluorescein will be measured and used to assess the relative potential for ocular irritancy of the test article(s).

7.1 Reagents

7.1.1 Hanks' Balanced Salt Solution with Ca^{++} and Mg^{++} (containing Penicillin/Streptomycin) (HBSS)

7.1.2 Fetal Bovine Serum (FBS)

7.1.3 Minimum Essential Medium (EMEM) without phenol red

7.1.4 Complete MEM without phenol red: EMEM without phenol red containing 1% FBS and 2 mM L-glutamine

7.1.5 Minimum Essential Medium (EMEM) with phenol red

7.1.6 Complete MEM with phenol red: EMEM with phenol red containing 1 % FBS and 2 mM L-glutamine (used for rinsing of test substances only)

7.1.7 Sodium Fluorescein – diluted in DPBS

7.1.8 Sterile Deionized Water

7.1.9 10% Buffered formalin solution

7.2 Environmental Conditions

Throughout this protocol, ranges for test material and test system exposure or incubation conditions (e.g., temperature, humidity, CO_2,) are presented. These ranges describe the equipment performance specifications under static conditions (i.e., in the absence of frequent opening of equipment doors, accessing chambers, changing loads, etc.), as presented in the relevant equipment SOPs.

7.3 Bovine Eyes

Bovine eyes will be obtained from the abattoir of J.W. TREUTH & SONS, Inc., Baltimore, MD. The eyes will be excised by an abattoir employee (as soon after slaughter as possible) and held in HBSS on ice. Once the required number of eyes has been obtained, the eyes will be transported to IIVS. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas will be initiated.

7.4 Preparation of Corneas

All eyes will be carefully examined for defects (opacity, scratches, pigmentation, etc.) and those exhibiting defects discarded. The tissue surrounding the eyeball will be carefully pulled away and the cornea will be excised leaving a 2 to 3 mm rim of sclera. The isolated corneas will be stored in a petri dish containing HBSS prior to mounting. Corneas will then be mounted in the corneal holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber will then be positioned on top of the cornea and tightened with screws.

The chambers of the corneal holder will then be filled with Complete EMEM without phenol red. The posterior chamber will always be filled first. The corneas will be incubated for the minimum of one hour at 32±1°C.

7.5 Sample Preparations

When appropriate, test articles will be diluted or suspended in either sterile deionized water or other Sponsor-directed solvent. Samples will be diluted on a w/v basis, unless otherwise specified by the Sponsor.

The stability of the test article under the storage conditions at the testing facility and under the actual experimental conditions will not be determined by Institute for In Vitro Sciences, Inc. (IIVS).

7.6 Treatment of Corneas

At the end of the one-hour incubation period, the medium will be removed from both chambers and replaced with fresh Complete MEM without phenol red. An initial opacity measurement will be performed on each of the corneas using an OP-KIT (Electro Design) opacitometer. The opacity of each cornea (including the negative control corneas) will be read against an air-filled chamber and recorded. Corneas that have an initial opacity reading that is greater than 7 will not be dosed. The medium will be removed from the anterior chamber and replaced with the test article, negative control, or positive control.

Protocol Attachment 1 will provide the test article designation(s), any preparation (including dilution and handling of the test material), the method to be used (if applicable), the length of the treatment, the post-treatment incubation time(s) and the applicable regulations to be followed.

7.6.1 Method A:

Liquids will generally be tested neat (undiluted), unless the Sponsor requests a specific dilution. Surfactants (either solids or liquids) will generally be tested at a 10% concentration in sterile deionized water unless otherwise directed by the Sponsor. Formulations will be tested after consultation with the Sponsor.

Seven hundred and fifty µL of test substance (test article, negative control or positive control) will be introduced into the anterior chamber. The holder will be slightly rotated (with the corneas maintained in a horizontal position) to ensure uniform distribution of the test substance over the cornea. Alternatively, the test material may be applied as a spray to cover the corneal surface. Spray application will be used only when directed by the Sponsor and will follow the specific procedure indicated. The test article and negative control treated corneas will be incubated at 32±1°C for 10 minutes or as directed by the Sponsor. The positive control will be incubated at 32±1°C for 10 minutes. On occasion, the negative control

exposure time may be selected to fit the longest test article exposure time of a test article run concurrently, but from an independent study. The test substance will then be removed and the epithelium will be washed at least 3 times (or until no visual evidence of test substance can be observed) with Complete MEM with phenol red. Once the media is free of test substance, the corneas will be given a final rinse with Complete MEM without phenol red. If the test article cannot be removed from the cornea a note will be documented in the raw data record. The anterior chamber will then be refilled with fresh Complete MEM without phenol red, and an opacity measurement will be performed. The corneas will then be incubated for approximately 2 hours at 32±1°C. At the completion of the incubation period, a second measure of opacity will be performed (final opacity). The values obtained at this second measurement will be used in calculating the corneal opacity.

7.6.2 Method B:

Solid materials will generally be tested as a 20% dilution (w/v) in sterile deionized water (or Sponsor directed solvent). Different concentrations may be evaluated at the Sponsor's request.

Seven hundred and fifty µL of test substance (test article, negative control or positive control) will be introduced into the anterior chamber. In some cases, the 20% (w/v) suspension may not be pipettable (e.g., test article floating in liquid), and a positive displacement pipet cannot be used. In those cases, a dosing spoon of the same approximate diameter of the exposed epithelium may be used to dose the corneas. Although it is understood that a 750 µL dose cannot be achieved, the corneas should be completely covered with the test article. The holder will be slightly rotated (with the corneas maintained in a horizontal position) to ensure uniform distribution of the test substance over the cornea. The corneas will be incubated in a horizontal position at 32±1°C for approximately 4 hours or as specified by the Sponsor. The test substance will then be removed and the epithelium washed at least 3 times (or until no visual evidence of test substance can be observed) with Complete MEM with phenol red. Once the media is free of test substance, the corneas will be given a final rinse with Complete MEM without phenol red. If the test article cannot be removed from the cornea a note will be recorded in the raw data record. The anterior and the posterior chambers will then be refilled with fresh Complete MEM without phenol red, and an opacity measurement performed immediately (without any further incubation)(final opacity).

7.7 Opacity Measurement

The opacitometer will determine the difference in the light transmission between each treated or control cornea and an air-filled chamber, and a numerical opacity value (arbitrary unit) will be displayed and recorded.

7.8 Permeability Determinations

Method A:

After the second opacity measurement is performed, the medium will be removed from both chambers of the holder. The posterior chamber will be refilled with fresh Complete MEM without phenol red. One mL of a 4 mg/mL sodium fluorescein solution will be added to the anterior chamber.
Method B:

After the opacity measurement is performed, the medium will be removed from the anterior chamber only and replaced with 1 mL of a 5 mg/mL sodium fluorescein solution.

After the addition of the fluorescein solution to the anterior chamber, the corneas will be incubated in a horizontal position for approximately 90 minutes at 32±1°C. The medium from the posterior chamber will be removed at the completion of the incubation period, and 360 µL will be transferred to the appropriate wells of a labeled 96-well plate. Three hundred and sixty µL of fresh Complete MEM without phenol red will be added to the wells designated as blanks. The optical density at 490 nm (OD_{490}) will be determined using a spectrophotometer. Samples reading 1.500 and above (OD_{490}) will be diluted to bring the reading within the linear range of the plater eader and the plate read again.

7.9 Fixation of the Corneas

After the medium is removed for the fluorescein determination, each cornea will be carefully removed from its holder and transferred to a prelabelled tissue cassette. The endothelial surface will be placed on sponge to protect it. The cassettes will be placed in 10% neutral buffered formalin and fixed for a minimum of 24 hours. Histological evaluation will not be performed, unless authorized by a protocol amendment. The fixed corneas will be stored at the testing facility for up to one year, and then disposed of unless otherwise instructed by the Sponsor. If the Sponsor elects to have histological evaluation performed, a directive will be added by amendment, and the corneas will be sent to the histology laboratory for processing.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The test will be accepted if the positive control produces an *In Vitro* Score that falls within two standard deviations of the historical mean.

9.0 EVALUATION OF TEST RESULTS

The change in opacity for each cornea (including the negative control corneas) will be calculated by subtracting the initial opacity reading from the final opacity reading. These values will then be corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value for each treatment

will be calculated by averaging the corrected opacity values of each cornea for a given treatment.

The mean OD_{490} for the blank wells will be calculated. The mean blank OD_{490} will be subtracted from the OD_{490} of each well (corrected OD_{490}). Any dilutions that are made to bring the OD_{490} values into the linear range of the platereader (OD_{490} should be less than 1.500), will have each diluted OD_{490} value multiplied by the dilution factor. The final corrected OD_{490} of the test article(s) and the positive control will be calculated by subtracting the average corrected OD_{490} of the negative control corneas from the corrected OD_{490} value of each treated cornea:

Final Corrected OD_{490} = (OD_{490} – mean blank OD_{490}) – average corrected negative control OD_{490}

The mean OD_{490} value of each treatment group will be calculated by averaging the final corrected OD_{490} values of the treated corneas for that treatment condition. Although the algorithms discussed are performed to calculate the final endpoint analysis at the treatment group level, the same calculations can be applied to the individual replicates.

9.1 *In Vitro* Score Calculation

The following formula will be used to determine the *In Vitro* Score:

In Vitro Score = Mean Opacity Value + (15 x Mean OD_{490} Value)

9.2 Data Interpretation

For regulatory purposes, the *In Vitro* Score (IVIS) cut-off values for identifying test chemicals as inducing serious eye damage (UN GHS Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) are found in the table below (OECD 437, adopted 26 July 2013). This guidance on categorization applies only to test article(s) evaluated using the appropriate standard protocols as described in OECD 437.

IVIS	UN GHS
≤3	No Category
>3; ≤55	No prediction can be made
>55	Category 1

For non-regulatory purposes, the following classification system was established by Sina et al. based on studies with a wide range of test materials. While this classification system provides a good initial guide to interpretation of these *in vitro* data, these specific ranges may not be applicable to all classes of materials or other exposure times. Whenever possible, results should be compared to "benchmark" materials tested under similar exposure conditions.

In Vitro Score:

≤ 25	= mild irritant
from 25.1 to 55	= moderate irritant
from 55.1 and above	= severe irritant

10.0 REPORT

A report of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. A summary will be presented for each treatment group. The report will also include a discussion of results. A copy of the protocol used for the study and any significant deviation(s) from the protocol will appear as a part of the final report.

11.0 RECORDS AND ARCHIVES

A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data, reports and specimens will be retained in the archives for a period of either a) 5 years, b) the length of time specified in the contract terms and conditions, or c) as long as the quality of the preparation affords evaluation, whichever is applicable.

12.0 TEST MATERIAL RETENTION

Unless indicated otherwise, all test articles provided by the sponsor and dose solutions used for testing or analysis before or during the course of the assay will be retained for one year after completion of the final report. These test articles and dose solutions may be disposed after this 1 year retention period according to IIVS SOP.

13.0 PROTOCOL AMENDMENTS

When it becomes necessary to change the approved protocol for a specific study, the change and the reason for it should be put in writing and signed by the Study Director as soon as practical. When the change may impact the study design and/or execution, verbal agreement to make this change should be made between the Study Director and Sponsor. This document is then provided to the Sponsor and is attached to the protocol as an amendment.

14.0 REFERENCES

Curren, R.D., Evans, M.G., Raabe, H.A., Ruppalt, R.R., Harbell, J.W. (2000) An histological analysis of damage to bovine corneas in vitro by selected ocular toxicants. **The Toxicologist** 54(1):188.

Gautheron, P., Dukic, M., Alix, D., and Sina, J.F. (1992) Bovine Corneal Opacity and Permeability Test: An *In Vitro* Assay of Ocular Irritancy. **Fundamental and Applied Toxicology** 18:442-449.

OECD (2013), *Test No. 437: Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing.
doi: 10.1787/9789264203846-en

Sina, J.F., Galer, D.M., Sussman, R.G., Gautheron, P.D., Sargent, E.V., Leong, B., Shah, P.V., Curren, R.D., and Miller, K. (1995) A collaborative evaluation of seven alternatives to the Draize eye irritation test using pharmaceutical intermediates. **Fundamental and Applied Toxicology** 26:20-31.

15.0 APPROVAL

IIVS STUDY DIRECTOR

26 April 2018
DATE

PROTOCOL ATTACHMENT 1

Test Article Preparation:	The test article will be tested neat. The test article was provided in a liquid nitrogen pressurized cylinder. An aliquot of the test article needed for the assay will be removed from the cylinder and placed into a glass vial just prior to dosing. After use, the remaining aliquoted sample will be discarded according to the disposal instruction in the Safety Data Sheet (SDS) provided by the Sponsor.
Test Method:	Method A
Test Article Exposure Time:	10 Minutes
Test Article Post Exposure Incubation:	120 Minutes
Histology:	Histological evaluation will not be performed at this time. If histological evaluation is to be performed, a directive will be added by a protocol amendment.

REGULATORY REQUIREMENTS:

Will this study be conducted according to **GLPs**? [**X**] **YES** or [ ] **NO**

If **YES**, please indicate which agency(ies) guidelines are to be followed:
[ ] FDA; [**X**] EPA TSCA; [ ] EPA FIFRA; [ ] OECD; [ ] Other

APPENDIX B

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

TA #	CORNEA #	INITIAL	FINAL	CHANGE	CORRECTED	AVG	STDEV
18AD37	40	1	2	1	1 0		
Neat	41	1	2	1	1 0		
10 minutes	42	2	2	0	0 0		
	43	1	2	1	1 0		
	46	1	2	1	1 0	0 8	0 4
Neg. Control	2	1	1	0	NA		
Sterile, DI water	3	1	1	0	NA		
10 minutes	4	1	1	0	NA	0 0	
Pos. Control	5	1	35	34	34 0		
Ethanol	6	1	24	23	23 0		
10 minutes	8	2	31	29	29 0	28 7	5 5

NA - Not Applicable

PERMEABILITY SCORE

Neg. Control
Sterile, DI water
10 minutes

Cornea #	OD490
2	0 005
3	0 007
4	0 009
Avg	0 007

Pos. Control
Ethanol
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
5	0 778	1	0 771
6	0 957	1	0 950
8	1 162	1	1 155
		Avg =	0 959
		STDEV=	0 192

18AD37
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
40	0 016	1	0 009
41	0 014	1	0 007
42	0 023	1	0 016
43	0 016	1	0 009
46	0 027	1	0 020
		Avg =	0 012
		STDEV=	0 006

IN VITRO SCORE

In Vitro Score = Mean Opacity Value + (15 x Mean OD490)

Test Article	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
18AD37	Neat	10 minutes	0 8	0 012	1 0
Ethanol	Neat	10 minutes	28 7	0 959	43 0

APPENDIX C

PHARMCO-AAPER
THE POWER OF THREE[3]

Certificate of Analysis
ETHYL ALCOHOL 200 PROOF, ABSOLUTE

Meets ACS/USP Grade Monographs
Grain Derived Ethanol
LOT # C16L0320P
QC # WO143117
Date of Manufacture: 09/27/17
Recommended Retest Date: Three Years from Date of Manufacture*
Main Catalog #: 111000200

This product meets or exceeds all specifications as set forth in the current ACS/USP monographs.

TEST	Monograph	SPECIFICATION	Results
Assay (by GC, corrected for water)	ACS	NLT 99.5%	99.98%
Assay (by specific gravity@15.56°C)	USP	NLT 99.5%	100.00%
Water (wt%)	ACS	NMT 0.2%	0.02%
Proof	27CFR 30.23	Lot Analysis	200.0
Identification A - Specific Gravity	USP	Meets the requirements of the test for Specific Gravity	Pass
Identification Test B	USP	Conforms to Infrared Spectra (Neat)	Pass
Color (APHA)	ACS	NMT 10	1
Clarity of Solution	USP	Sample Solution A & B show the same clarity as that of water, or their opalescence is not more pronounced than that of Standard suspension A	Pass
Color of Solution	USP	The Sample Solution has the appearance of water or is not more intensely colored than the Standard solution	Pass
Solubility in Water	ACS	To Pass Test	Pass
Specific Gravity	USP	NMT 0.7962 at 15.56C	0.7936
Residue on Evaporation	ACS	NMT 0.001%	0.000%
Limit of Nonvolatile Residue	USP	The weight of the residue does not exceed 2.5mg	0.0 mg
Acetone/Isopropyl Alcohol	ACS	To Pass Tests	Pass
Titrable Acid	ACS	NMT 0.0005 meq/g	0.0001 meq/g
Titrable Base	ACS	NMT 0.0002 meq/g	0.0001 meq/g
Acidity or Alkalinity	USP	The solution is pink (30ppm, expressed as acetic acid)	Pass
Methanol	ACS	NMT 0.1%	< 0.1%
Substances Darkened by Sulfuric Acid	ACS	To Pass Test	Pass
Substances Reducing Permanganate	ACS	To Pass Test	Pass
Ultraviolet Absorbance	USP	NMT 0.40 at 240 nm NMT 0.30 between 250 nm and 260 nm NMT 0.10 between 270 nm and 340 nm The spectrum shows a steadily descending curve with no observable peaks or shoulders	0.26 0.10 0.02 Pass
Organic Impurities	USP	Methanol NMT 200 ppm Acetaldehyde and Acetal NMT 10ppm Benzene NMT 2ppm Sum of all other impurities NMT 300ppm	None Detected None Detected None Detected 2 ppm

Form: Ethanol, Pure, 200, ACS/USP, #101, Rev 5.0, 06/16, KAD

Approved by: K. Ryan, Quality Control Chemist

Disclaimer: For Industrial/Lab use only. Not intended as a Drug Substance, Medical Device or Disinfectant. Appropriate/legal use of this product is the responsibility of the user. *Excluding UV/Vis for pure Ethyl Alcohol (See shelf life statement). (Rev. # disclaimer only, rev 3.6, 07/15, PD)

PHARMCO-AAPER: 58 Vale Road, Brookfield, CT 06804. 1.800.243.5360

Quality Biological
MAKING SOLUTIONS WORK

PRODUCT: WATER – Ultra Pure
(Cell Culture Grade)

CATALOG#: 118-162-101 Size: 500ml

LOT#: 721803

CERTIFICATE OF ANALYSIS

This product is de-ionized by reverse osmosis and sterile filtered.

	Specifications	Result
RESISTIVITY:	15 – 20 Meg Ohms/cm	17.90 Meg Ohms/cm
ENDOTOXIN:	Less than 0.06 Eu/ml	pass
APPEARANCE:	Clear colorless fluid.	pass
STERILITY:	No evidence of bacterial or fungal contamination after 14 days testing on Fluid Thioglycollate and Tryptic Soy Broth.	pass

RECOMMENDED STORAGE CONDITIONS: 15 – 30°C

MANUFACTURED DATE: 08/2016

EXPIRATION DATE: 08/2018

10/7/2016

Quality Assurance Date

FOR RESEARCH USE ONLY
NOT FOR IN-VITRO DIAGNOSTIC USE.

7581 Lindbergh Drive • Gaithersburg, MD 20879
(301) 840-9331 • Toll Free (800) 443-9331 • Fax (301) 840-0743
Email: customerservice@qualitybiological.com • Web Site: www.qualitybiological.com

C4

charles river

FINAL REPORT

Test Facility Study No. 20146044

Sponsor Study No.

DEREK Prediction on Skin Sensitization of

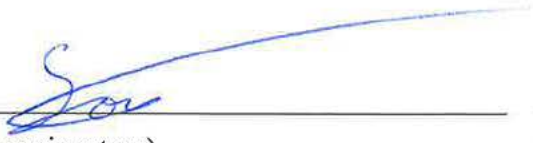
TEST FACILITY:
Charles River Laboratories Den Bosch BV
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

TABLE OF CONTENTS

LIST OF APPENDICES

AUTHENTICATION STATEMENT

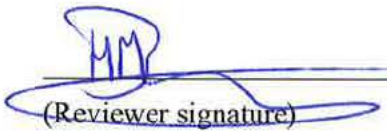
Herewith the author declares that the structures used are the structures provided by the sponsor.



28-Mar-2018

(Author signature) (Date)

S. Jonis, MSc
Regulatory Affairs Assistant
Charles River Laboratories Den Bosch BV



28- Mar- 2018

(Reviewer signature) (Date)

H.M. Barentsen, PhD
Senior Regulatory Toxicologist
Charles River Laboratories Den Bosch BV

1. RESPONSIBLE PERSONNEL

1.1. Test Facility

Test Facility	Charles River Laboratories Den Bosch BV Hambakenwetering 7 5231DD ‘s-Hertogenbosch The Netherlands

2. INTRODUCTION

The objective of this study was to obtain a prediction on the potential for skin sensitization of the test item with the in silico model DEREK NEXUS. In this assessment version 6.0.1 of DEREK NEXUS was used.

3. BACKGROUND/SCOPE

DEREK NEXUS is a knowledge-based system that contains 90 alerts for skin sensitization based on the presence of molecular substructures. LHASA (see Appendix I) has inserted validation comments for the skin sensitization alerts.

The level of likelihood of a structure being sensitizing to skin is expressed in terms of:

Certain	There is proof that the proposition is true.
Probable	There is at least one strong argument that the proposition is true and there are no arguments against it.
Plausible	The weight of evidence supports the proposition.
Equivocal	There is an equal weight of evidence for and against the proposition.

The default of DEREK NEXUS for the level of likelihood, mentioning all alerts which are evaluated as being equivocal or greater was used in this assessment.
If a substance is predicted to be no skin sensitizer, DEREK NEXUS contains an expert-derived functionality to provide negative predictions for skin sensitization. This functionality further evaluates those compounds which do not fire any skin sensitization alerts in DEREK NEXUS. The query compound is compared to a Lhasa reference set of Ames test or skin sensitization data, producing the following outcomes:

- In compounds where all features in the molecule are found in accurately classified compounds from the reference set, a negative prediction is displayed: inactive.
- For those query compounds where features in the molecule are found in non-alerting skin sensitizers in the Lhasa reference set, the prediction remains negative and the misclassified[1] features are highlighted to enable the negative prediction to be verified by expert assessment.
- In cases where features in the molecule are not found in the Lhasa reference set, the prediction remains negative and the unclassified[2] features are highlighted to enable the negative prediction to be verified by expert assessment.

If a substance is predicted to be a skin sensitizer, its potency is predicted by DEREK NEXUS by calculating an EC3 value based on experimental data from the closest structurally-related substances (at least 3 substances should be present) using the following equation:

[1] Misclassified features are those that have been derived from non-alerting mutagens in the Lhasa Ames test reference set
[2] Unclassified features are those that have not been found in the Lhasa Ames test reference set

$EC3_Q = MW_Q / (\Sigma \omega_{NN} / \Sigma T_{NN})$

MW = molecular weight
T = Tanimoto similarity score
ω = weighting factor = $(MW_{NN}/EC3) * T_{NN}$
Q = query compound
NN = nearest neighbour

The EC3 is the estimated concentration needed to produce a stimulation index of 3.

4. MATERIALS AND METHODS

4.1. Test Item

Identification:

Chemical name:

CAS Number:

Molecular weight:

Molecular formula:

Chemical structure:

5. RETENTION OF RECORDS

The final report generated by Charles River from this study will be transferred to a Charles River archive no later than the date of final report issue.

6. RESULTS AND CONCLUSION

The result as generated by DEREK NEXUS is presented in Appendix 2. The relevant QSAR Model Reporting Format (QMRF) and the QSAR Prediction Reporting Format (QPRF) are presented in Appendix 3.

DEREK NEXUS version 6.0.1 did not match the query structure with any structural alerts or examples for skin sensitization. However, the query structure contains features that were not found in the Lhasa skin sensitization negative prediction dataset (unclassified).
is predicted to be not sensitizing to the skin, but this prediction should be considered with caution.

Appendix 1
Disclaimer

Disclaimer

Lhasa Limited makes no warranties, either expressed or implied, regarding the DEREK NEXUS software, its merchantability, or its fitness for any particular purpose. In no event will Lhasa be liable for any special, consequential, indirect or similar damages including any loss of profits or lost data arising out of the use of the DEREK NEXUS software.

LHASA Limited, Granary Wharf House, 2 Canal Wharf, LEEDS, LS11 5PS, United Kingdom
Tel: ++44 (0) 113 394 6020 E-mail: info@lhasalimited.org

Appendix 2

Lhasa Limited

Derek nexus

Derek Nexus Report

Report Information

Author	Report date	Prediction date	Program version
cr209308	05 March 2018 14:02:55	05 March 2018 13:35:22	Derek Nexus: 6.0.1, Nexus: 2.2.1

Processing Options

Selected Species	Selected Knowledge Base(s)	Reasoning Level
mammal	Derek KB 2018 1.1	At least EQUIVOCAL
Perceive tautomers	**Perceive mixtures**	**Match alerts without rules**
Yes	Yes	No
Show Open likelihood	**Show Negative Predictions**	**Show Rapid Prototypes**
No	Yes	Yes
Filter nearest neighbours on misclassified features		
Yes		

Predictions

Knowledge Base: Derek KB 2018 1.1

Version	Last Modified Date	Certified by
1.1	23/11/2017 10:38:05	Lhasa Limited, Leeds, Yorkshire, UK

Reasoning Summary

◆ **Skin sensitisation in mammal is NON-SENSITISER**
- Contains unclassified features

Endpoints not firing any alerts at the selected reasoning level (0)

Alert Descriptions

No alerts fired

Reasoning Details

Skin sensitisation in mammal is NON-SENSITISER (from KB: Derek KB 2018 1.1)

The parameters that have influenced your prediction are: substructures in the input structure, which have the potential to cause skin sensitisation; your selected species, which is mammal.

Details
The query structure contains features (highlighted in the structure panel) that were not found in the Lhasa skin sensitisation negative prediction dataset and do not match any structural alerts or examples for skin sensitisation in Derek. It is predicted to be a non-sensitiser.

Appendix 3
QMRF and QPRF DEREK NEXUS Skin Sensitization

QMRF as prepared by LHASA Ltd (UK)

QMRF CHAPTER TITLES	SECTION TITLES	MODEL DESCRIPTION (Fill in this column)
1. QSAR identifier		
	1.1. QSAR identifier (title):	Derek Nexus - skin sensitisation
	1.2. Other related models:	Derek Nexus contains alerts for multiple endpoints, including mutagenicity, chromosome damage, carcinogenicity, hepatotoxicity, teratogenicity and skin irritation
	1.3. Software coding the model:	Derek Nexus v6.0 contains 80 alerts for skin sensitisation, together with reasoning rules encoding physicochemical descriptors. In addition to a prediction of skin sensitisation potency for alerting query compounds, Derek evaluates potentially misclassified and unclassified features in compounds that do not activate skin sensitisation alerts or examples
2. General information		
	2.1. Date of QMRF:	26 July 2018
	2.2. QMRF author(s) and contact details:	Kate Langton, Lhasa Limited, 22-23 Blenheim Terrace, Woodhouse Lane, Leeds, LS2 9HD, UK
	2.3. Date of QMRF update(s):	1 December 2017
	2.4. QMRF update(s):	Lisa Fish, Lhasa Limited, Granary Wharf House, 2 Canal Wharf, Leeds, LS11 5PS, UK 1.3, 2.3, 2.4, 2.6, 3.4, 4.1, 4.2, 4.4, 4.5, 4.6, 5.1, 5.2
	2.5. Model developer(s) and contact details:	Lhasa Limited, Granary Wharf House, 2 Canal Wharf, Leeds, LS11 5PS
	2.6. Date of model development and/or publication:	Derek Nexus 6.0 was released on 12 December 2017
	2.7. Reference(s) to main scientific papers and/or software package:	[1] Sanderson DM & Earnshaw CG (1991). Computer prediction of possible toxic action from chemical structure; The DEREK system. Human and Experimental Toxicology 10, 261-273. [2] Judson PN, Marchant CA & Vessey JD (2003). Using argumentation for absolute reasoning about the potential toxicity of chemicals. Journal of Chemical Information and Computer Sciences 43, 1364-1370. [3] Marchant CA, Briggs KA & Long A (2008). In silico tools for sharing data and knowledge on toxicity and metabolism: Derek for Windows, Meteor, and Vitic. Toxicology Mechanisms and Methods 18, 177- 187. [4] Judson PN, Stalford SA & Vessey J (2013). Assessing confidence in predictions made by knowledge-based systems. Toxicology Research 2, 70-79
	2.8. Availability of information about the model:	Derek Nexus is a proprietary, rule-based expert system for the prediction of toxicity. Its knowledge base is composed of alerts, examples and reasoning rules which may each contribute to the predictions made by the system. Each alert in Derek describes a chemical substructure believed to be responsible for inducing a specific toxicological outcome (often referred to as a toxicophore). Alerts are derived by experts, using toxicological data and information regarding the biological mechanism of action. Where relevant, metabolism data may be incorporated into an alert, enabling the prediction of compounds which are not directly toxic but are metabolised to an active species. The derivation of each alert is described in the alert comments along with supporting references and example compounds where possible. By supplying this information to the user, Derek provides highly transparent predictions. The use of structural alerts for the prediction of toxicity is both widely understood and the subject of many publications.
	2.9. Availability of another QMRF for exactly the same model:	No
3. Defining the endpoint - OECD Principle 1		
	3.1. Species:	Predictions are made for the class of mammals and can be broken down into species (e.g. mouse, human, guinea pig)
	3.2. Endpoint:	4 Human health effects 4.6 Skin sensitisation
	3.3. Comment on endpoint:	The Derek Nexus model for skin sensitisation is developed from several sources of data. Sources of primary data used for alert development include [1] guinea pig data, such as the Buehler and maximisation tests, [2] human data from maximisation and patch tests, [3] mouse data, mostly from the local lymph node assay. Secondary data sources of toxicity such as [4] EpiVs categories and [5] R43 classifications have also been used. Additionally, alert writers consider both mechanistic evidence and chemical properties (such as reactivity).
	3.4. Endpoint units:	Derek Nexus makes predictions for and against toxicity through reasoning. For the endpoint of skin sensitisation, predictions for toxicity decrease in confidence in the following order: certain> probable>plausible>equivocal. Predictions against toxicity increase in confidence in the following order: inactive (with unclassified and/or misclassified features)>inactive>improbable. These likelihood levels have been shown to correlate with predictivity (Judson et al, 2003). Multiple data sources (e.g. toxicity data from multiple assays and mechanistic evidence) are synthesised into the structure-activity relationships that underpin Derek Nexus predictions. An appreciation of the assay units applied by alert writers when building the alert training set. However, predictions are not restricted to a specific assay and, as a result, do not include units.
	3.5. Dependent variable:	Data from several toxicity assays (e.g. LLNA, GPMT, HRIPT) and mechanistic studies (e.g. DPRA) are synthesised to arrive at an expert conclusion of whether compounds within the model training set is likely to be a skin sensitiser.
	3.6. Experimental protocol:	The model is based primarily on data from Guinea Pig Maximisation Test or Local Lymph Node Assay conducted following standard test protocol (GPMT: OECD Test Guideline 406, LLNA: OECD Test Guideline 429). If activity is observed in a non-standard assay or protocol this will be mentioned in the comments. The process of alert development for skin sensitisation has been published [Langton et al]
	3.7. Endpoint data quality and variability:	Alert writers use all available and relevant information in the public domain (and proprietary data, where available) for alert development. Wherever possible, primary references are used as data sources. (i) the data are subject to expert assessment prior to inclusion in the alert training set using, amongst other criteria, OECD test guidelines and (ii) the references themselves are cited in the alert comments enabling users to conduct their own expert assessments on data quality
4. Defining the algorithm - OECD Principle 2		
	4.1. Type of model:	Expert derived structural alerts for skin sensitisation (2D SARs), physicochemical properties and associated reasoning. Following alert evaluation, Derek will make a prediction of skin sensitisation potency for alerting query compounds, where possible. This is based on the activity (EC3 values) for nearest neighbours derived from a local lymph node assay data set. In addition Derek evaluates whether non-alerting query compounds contain any features that are either (i) also present in non-alerting skin sensitisers in a large Skin Sensitisation reference set (misclassified features) or (ii) not present in a large Skin Sensitisation reference set (unclassified features).
	4.2. Explicit algorithm:	[1] Structural alerts, [2] logic of argumentation, [3] nearest neighbours (within same alert as query compound) based on Tanimoto similarity, and [4] feature based database search
	4.3. Descriptors in the model:	[1] Markush structures encoding activating and deactivating features (known as patterns in the Derek Nexus knowledge base), [2] 2D structural fingerprints.
	4.4. Descriptor selection:	There is as a priori assumption that patterns, physicochemical properties and associated reasoning will be used to model toxicity within Derek Nexus. Further, experts identified that 1) predictions of potency (LLNA EC3) could be made using nearest neighbours within the same alert, and 2) misclassified and unclassified features were useful descriptors for determining the reliability of negative predictions for non-alerting compounds
	4.5. Algorithm and descriptor generation:	Alert writers design the patterns to describe the activating and deactivating features found during expert assessment of the alert training set. Structural fingerprints are generated from the 409 compounds in the local lymph node data set. Misclassified and unclassified features are generated by processing a large Skin Sensitisation reference set (comprising 1,386 sensitisers and 1420 non-sensitisers) against Derek Nexus (v6.0) and fragmenting
	4.6. Software name and version for descriptor generation:	Alert writers use the Derek Knowledge Editor (v2.0) for the implementation of patterns. Structural fingerprints and fragmentation are generated using an in-house algorithm
	4.7. Chemicals/Descriptors ratio:	This is not applicable as the structural alerts are knowledge-based rather than statistically based
5. Defining the applicability domain - OECD Principle 3		
	5.1. Description of the applicability domain of the model:	The scopes of the structure-activity relationships describing the skin sensitisation endpoint are defined by the developer to be the applicability domain for the model. Therefore, if a chemical activates an alert describing a structure activity for skin sensitisation it can be considered to be within the applicability domain. The applicability of potency predictions may be judged, and modified, by the user based on the displayed data for nearest neighbours. If a compound does not activate an alert or reasoning rule then Derek makes a negative prediction. The applicability of the negative prediction to the query compounds can be determined by an expert, if required, by investigating the presence (or absence) of misclassified and/or unclassified features.

	6.2. Method used to assess the applicability domain:	The applicability domain of each alert is defined by the alert developer on the basis of the training set data and expert judgement on the chemical and biological factors which affect the mechanism of action for each alert. For potency predictions, at least three nearest neighbours are required within alerting space to make a prediction. For non-alerting compounds, users should determine the applicability of negative predictions by evaluating the information supplied by Derek (i.e. the presence or absence of modulated and/or unclassified features).
	6.3. Software name and version for applicability domain assessment:	This is not applicable.
	6.4. Limits of applicability:	Limits for individual alerts are mainly defined by restrictions in the scope of the alerts which are available for inspection within the software.
6. Internal validation - OECD Principle 4		
	6.1. Availability of the training set:	Non-proprietary elements of the training set are available through the references, and illustrated by the examples, within Derek Nexus. The illustrative examples are not available, due to the proprietary nature of Derek Nexus.
	6.2. Available information for the training set:	CAS RN: No Chemical name: No Smiles: No Formula: No InchI: No MOL file: No
	6.3. Data for each descriptor variable for the training set:	This is not applicable.
	6.4. Data for the dependent variable for the training set:	This is not applicable.
	6.5. Other information about the training set:	This is not applicable.
	6.6. Pre-processing of data before modelling:	This is not applicable.
	6.7. Statistics for goodness-of-fit:	This is not applicable.
	6.8. Robustness - Statistics obtained by leave-one-out cross-validation:	This is not applicable.
	6.9. Robustness - Statistics obtained by leave-many-out cross-validation:	This is not applicable.
	6.10. Robustness - Statistics obtained by Y-scrambling:	This is not applicable.
	6.11. Robustness - Statistics obtained by bootstrap:	This is not applicable.
	6.12. Robustness - Statistics obtained by other methods:	This is not applicable.
7. External validation - OECD Principle 4		
	7.1. Availability of the external validation set:	External validation is carried out on each knowledge base release. The data sets used for validation are available in the public domain, but the curated versions used at Lhasa are proprietary, so are not made available.
	7.2. Available information for the external validation set:	CAS RN: No Chemical name: No Smiles: No Formula: No InchI: No MOL file: No
	7.3. Data for each descriptor variable for the external validation set:	This is not applicable.
	7.4. Data for the dependent variable for the external validation set:	This is not applicable.
	7.5. Other information about the external validation set:	Three published data sets have been used for alert validation: [1] Cronin and Basketter, [2] Gerberick et al and Kern et al and [3] a collection of local lymph node assay data for 137 compounds published in Contact Dermatitis which have been extracted from Vitic Nexus (13 September 2012). Further, the relationship between likelihood levels and prediction accuracy has been assessed (Judson et al 2013). Finally, several external evaluations have been published [Ronja et al., Nukada et al].
	7.6. Experimental design of test set:	Proprietary data sets were sought.
	7.7. Predictivity - Statistics obtained by external validation:	The software reports the number of positive and negative compounds from the validation data sets that activate each alert and calculates positive predictivity using this data.
	7.8. Predictivity - Assessment of the external validation set:	Data derived from LLNA and guinea pig assays covering 516 unique compounds. The compounds in the data sets are primarily small chemicals and so are representative of the structures used to build the model.
	7.9. Comments on the external validation of the model:	This is not applicable.
8. Providing a mechanistic interpretation - OECD Principle 5		
	8.1. Mechanistic basis of the model:	All alerts describing structure-activity relationships for the skin sensitisation endpoint have a mechanistic basis wherever possible. Mechanistic information is detailed in the comments associated with an alert and can include information on both the mechanism of action and biological target.
	8.2. A priori or a posteriori mechanistic interpretation:	The mechanistic basis of the model was developed a priori by examining the toxicological and mechanistic evidence before developing the structure-activity relationship.
	8.3. Other information about the mechanistic interpretation:	All references supporting the mechanistic basis of an alert are detailed and available for inspection within the software.
9. Miscellaneous information		
	9.1. Comments:	Derek Nexus may be used to assess the toxicity of a wide range of chemical classes, including food, drug, cosmetic, and industrial chemicals, and the system provides predictions for over 50 toxicological endpoints, including mutagenicity, chromosome damage, carcinogenicity, skin sensitisation and reproductive toxicity. Skin sensitisation predictions from Derek have shown potential utility when used as part of a weight of evidence assessments [Dreidoi et al] and integrated testing strategies for skin sensitisation [Ronja et al, Nukada et al]. Additionally, Derek mutagenicity predictions are submitted as part of the regulatory requirements on genotoxic impurities in pharmaceuticals [Sutter et al]
	9.2. Bibliography:	[1] Sanderson DM & Earnshaw CG (1991). Computer prediction of possible toxic action from chemical structure. The DEREK system. Human and Experimental Toxicology 10, 261-273. [2] Judson PN, Marchant CA & Vessey JD (2003). Using argumentation for absolute reasoning about the potential toxicity of chemicals. Journal of Chemical Information and Computer Sciences 43, 1364-1370. [3] Marchant CA, Briggs KA & Long A (2008). In silico tools for sharing data and knowledge on toxicity and metabolism: Derek for Windows, Meteor, and Vitic. Toxicology Mechanisms and Methods 18, 177-187. [4] Judson PN, Stalford SA & Vessey J (2013). Assessing confidence in predictions made by knowledge-based systems. Toxicology Research 2, 70-79. [5] Langham K, Pallewicz GY, Long A, Marchant CA, Basketter DA (2008). Structure-activity relationships for skin sensitization: recent improvements to Derek for Windows. Contact Dermatitis 58, 342-347. [6] Potts RO and Guy RH (1992). Predicting skin permeability. Pharmaceutical Research 9, 663-669. [7] Cronin MT & Basketter DA (1994). Multivariate QSAR analysis of a skin sensitization database. SAR and QSAR in Environmental Research 2, 159-179. [8] Gerberick GF, Ryan CA, Kern PS, Schlatter H, Dearman RJ, Kimber I & Patlewicz GY, Basketter DA (2005). Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. Dermatitis 16, 157-202. [9] Kern PS, Gerberick GF, Ryan CA, Kimber I, Aptula A & Basketter DA (2010). Local lymph node data for the evaluation of skin sensitization alternatives: a second compilation. Dermatitis 21, 5-32. [10] Dreidoi C, Autry P, Abe N, Alépée N, Aptula A, Araki D, Dufour E, Dürrour N, Hibatallah J, Keller D, Kern P, Kral A, Marrec-Fairley M, Maxwell G, Rowland J, Safford B, Schellauf F, Schepky A, Swanson C, Tailefert T, Tessier N, Tessier S, Weltjen HU, Wikler P & Schwarz J (2012). Guiding principles for the implementation of non-animal safety assessment approaches for cosmetics: Skin sensitisation. Regulatory Toxicology and Pharmacology 63, 40-52. [11] Ronja E, Attenberg T, Buist H, Kreiser D & Schürmann G (2013). The OSIRIS Weight of Evidence approach: IES for skin sensitisation. Regulatory Toxicology and Pharmacology 67, 146-156. [12] Nukada Y, Miyazawa M, Kazutoshi S, Sakaguchi H & Nishiyama N (2013). Data integration of non-animal tests for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. Toxicology in Vitro 27, 609-618. [13] Sutter A, Amberg A, Boyer S, Briggs A, Contrera JF, Custer LL, Dobo KL, Gerets V, Glowienke S, van Gompel J, Greene N, Muster W, Nicolette J, Reddy MV, Thybaud V, Vock E, White AT & Müller L (2013). Use of in silico systems and expert knowledge for structure-based assessment of potentially mutagenic impurities. Regulatory Toxicology and Pharmacology 67, 35-52.
	9.3. Supporting information:	No information is available.
10. Summary (JRC Inventory)		
	10.1. QMRF number:	Not by JHRC
	10.2. Publication date:	Not by JHRC
	10.3. Keywords:	Not by JHRC
	10.4. Comments:	Not by JHRC

QPRF

1. Substance

1.2 EC number: Not available

1.3 Chemical name:

1.4 Structural formula:

1.5 Structure codes:

d. Stereochemical features: DEREK prediction does take stereochemistry into account, if evidence of its influence on reactivity has been presented in literature; otherwise only 2D connectivity is taken into consideration. In this molecule no stereochemistry is present.

2. General information

2.1 Date of QPRF: 03 March 2018.

2.2 QPRF author and contact details: Charles River Laboratories Den Bosch

3. Prediction

3.1 Endpoint (OECD Principle 1)

a. Endpoint: Skin sensitization.

b. Dependent variable: Data from several toxicity assays (e.g. LLNA, GPMT, HRIPT) and mechanistic studies (e.g. DPRA) are synthesised to arrive at an expert conclusion of whether compounds within the model training set are likely to be a skin sensitizer.

3.2 Algorithm (OECD Principle 2)

a. Model or submodel name: DEREK NEXUS – skin sensitization.

b. Model version: DEREK NEXUS 6.0.1.

c. Reference to QMRF: The QMRF DEREK NEXUS 6.0 – skin sensitization was prepared by the provider LHASA Ltd (UK).

d. Predicted value (model result): Assessment with DEREK NEXUS did not yield any skin sensitization alerts for this structure.

e. Predicted value (comments): Not relevant; the structure did not fall within one of the currently 90 skin sensitization alerts, and consequently the structure is predicted to be negative. The structure contains no misclassified features, but does contain unclassified features and therefore the prediction should be considered with caution.

f. Input for prediction: See section 1.5.

g. Descriptor values: Not relevant.

3.3 Applicability domain (OECD principle 3)

a. Domains:

i. *descriptor domain:* see QMRF section 5.1

ii. *structural fragment domain:* for skin sensitization, which features multiple alerts believed to cover most of the mechanisms and chemical classes responsible for activity, "no alerts fired" may be extrapolated to a negative prediction. However, the substructure "trifluorovinyloxy" could not be found in the Lhasa skin sensitization negative prediction dataset. Therefore, this prediction should be considered with caution.

iii. *mechanism domain*: as the prediction is "no alerts fired" none of the mechanisms for skin sensitization is predicted to be applicable to this structure.

iv. *metabolic domain:* not relevant.

b. Structural analogues: not applicable.

c. Considerations on structural analogues: Not applicable

3.4 The uncertainty of the prediction (OECD principle 4)

DEREK NEXUS predictive performance against a combined human dataset had an accuracy of 77%.
https://www.lhasalimited.org/Public/Library/2014/Derek%20Nexus%20predicts%20human%20skin%20sensitisation%20accurately.pdf

The structure did not match any structural alerts or examples for skin sensitization in DEREK; however, the substructure "trifluorovinyloxy could not be found in the Lhasa skin sensitization negative prediction dataset. Therefore, this prediction should be considered with caution.

3.5 The chemical and biological mechanisms according to the model underpinning the predicted result (OECD principle 5).

Not relevant, DEREK NEXUS did not yield any alerts for skin sensitization.

4. Adequacy (Optional)

4.1 **Regulatory purpose:** The present prediction may be used for preparing the REACH Registration Dossier on the substance for submission to ECHA, as required by Regulation (EC) 1907/2006 and related amendments.

4.2 **Approach for regulatory interpretation of the model result:** This result can be directly used within a weight-of-evidence approach to complete the endpoint skin sensitization.

4.3 **Outcome:** Substance should not be classified according to DEREK NEXUS; however, this (Q)SAR prediction cannot be used as stand-alone for classification purposes or for covering the endpoint skin sensitization for registration under REACH.

4.4 **Conclusion:** The result is adequate to be used in a weight-of-evidence approach together with in chemico/in vitro studies to complete the endpoint skin sensitization.

charles river

FINAL REPORT

Test Facility Study No. 20146046

Sponsor Reference No.

In Chemico Determination of the Skin Sensitization Potential of using the Direct Peptide Reactivity Assay (DPRA)

TEST FACILITY:
Charles River Laboratories Den Bosch BV
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

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QUALITY ASSURANCE STATEMENT

Study title: In Chemico Determination of the Skin Sensitization Potential of using the Direct Peptide Reactivity Assay (DPRA).

This report was inspected by the Test Facility Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s). The reported method and procedures were found to describe those used and the report reflects the raw data. The Test Facility inspection program was conducted in accordance with Standard Operating Procedure. During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Test Facility Study No.	20146046			
Type of Inspections	**Phase/Process**	**Start Inspection date**	**End Inspection date**	**Reporting date to TFM and SD***
Study	Final Study Plan	30-Mar-2018	30-Mar-2018	30-Mar-2018
	Report	01-Jun-2018	01-Jun-2018	01-Jun-2018
	Final Report	08-Jun-2018	08-Jun-2018	08-Jun-2018
Process	**In Vitro ADME** Test Item Handling Exposure Observations/Measurements Specimen Handling	22-Jan-2018	02-Feb-2018	05-Feb-2018
	Test Item Receipt Test Item Handling	13-Mar-2018	21-Mar-2018	21-Mar-2018

*TFM=Test Facility Management SD = Study Director
The review of the final report was completed on the date of signing this QA statement.

Date: 13 June 2018

Marjolijn Kock, MSc
Quality Assurance Auditor

COMPLIANCE STATEMENT AND REPORT APPROVAL

The study was performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America, Japan, and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Exceptions from the above regulations are listed below.

- Concentration, stability, and homogeneity of test item formulations were not determined in this study. However, to limit the impact, the test item preparation was performed with approved procedures and documented in detail. Preparations were visually inspected for homogeneity prior to use and all preparations were used within 4 hours after preparation of the formulation.

This study was conducted in accordance with the procedures described herein. All deviations authorized/acknowledged by the Study Director are documented in the Study Records. The report represents an accurate and complete record of the results obtained.

There were no deviations from the above regulations that affected the overall integrity of the study or the interpretation of the study results and conclusions.

Date: 14 June 2018

J. Reinen, PhD, ERT

Study Director

1. RESPONSIBLE PERSONNEL

1.1. Test Facility

Study Director	J. Reinen, PhD, ERT
Test Facility Management	E.J. van de Waart, MSc, ERT Head of Discovery & Environmental Sciences

2. SUMMARY

The objective of this study was to determine the reactivity of towards model synthetic peptides containing either cysteine (SPCC) or lysine (SPCL). After incubation of the test item with either SPCC or SPCL, the relative peptide concentration was determined by High-Performance Liquid Chromatography (HPLC) with gradient elution and photodiode array (PDA) detection at 220 nm and 258 nm. SPCC and SPCL Percent Depletion Values were calculated and used in a prediction model which allows assigning the test item to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

The study procedures described in this report were based on the most recent OECD guideline.

Isopropanol was found to be an appropriate solvent to dissolve the test item and was therefore used in this Direct Peptide Reactivity Assay (DPRA) study. An overview of the obtained assay validation parameters is presented in the table below.

Acceptability of the Direct Peptide Reactivity Assay (DPRA)

	Cysteine reactivity assay		Lysine reactivity assay	
	Acceptability criteria	Results for SPCC	Acceptability criteria	Results for SPCL
Correlation coefficient (r^2) standard calibration curve	>0.99	0.998	>0.99	0.998
Mean peptide concentration RC-A samples (mM)	0.50 ± 0.05	0.470 ± 0.020	0.50 ± 0.05	0.485 ± 0.007
Mean peptide concentration RC-C samples (mM)	0.50 ± 0.05	0.491 ± 0.037	0.50 ± 0.05	0.484 ± 0.003
Mean peptide concentration RC-$C_{isopropanol}$ samples (mM)	0.50 ± 0.05	0.506 ± 0.013	0.50 ± 0.05	0.501 ± 0.004
CV (%) for RC samples B and C	<15.0	5.0	<15.0	1.0
Mean peptide depletion cinnamic aldehyde (%)	60.8-100	73.6	40.2-69.0	55.0
SD of peptide depletion cinnamic aldehyde (%)	<14.9	2.0	<11.6	0.1
SD of peptide depletion for the test item (%)	<14.9	5.3	<11.6	0.4

RC = Reference Control; CV = Coefficient of Variation; SD = Standard Deviation.

The validation parameters, i.e. calibration curve, mean concentration of Reference Control (RC) samples A, C and $C_{isopropanol}$, the CV for RC samples B and C, the mean percent peptide depletion values for the positive control with its standard deviation value and the standard deviation value of the peptide depletion for the test item, were all within the acceptability criteria for the DPRA.

Upon preparation as well as after incubation of the SPCC test item samples, no precipitate or phase separation was observed in any of the samples. Upon preparation as well as after incubation of the SPCL test item samples, a precipitate was observed.

An overview of the individual results of the cysteine and lysine reactivity assays as well as the mean of the SPCC and SPCL depletion are presented in the table below. In the cysteine reactivity assay the test item showed 3.1% SPCC depletion while in the lysine reactivity assay the test item showed 0.3% SPCL depletion. The mean of the SPCC and SPCL depletion was 1.7% and as a result the test item was considered to be negative in the DPRA and classified in the “no or minimal reactivity class” when using the Cysteine 1:10 / Lysine 1:50 prediction model.

SPCC and SPCL Depletion, DPRA Prediction and Reactivity Classification for the Test Item

Test item	SPCC depletion		SPCL depletion		Mean of SPCC and SPCL depletion	DPRA prediction and reactivity classification
	Mean	± SD	Mean	± SD		Cysteine 1:10 / Lysine 1:50 prediction model
	3.1%	±5.3%	0.3%	±0.4%	1.7%	Negative: No or minimal reactivity

SD = Standard Deviation.

In conclusion, since all acceptability criteria were met this DPRA is considered to be valid. was negative in the DPRA and was classified in the "no or minimal reactivity class" when using the Cysteine 1:10 / Lysine 1:50 prediction model. However, since precipitation was observed after the incubation period for SPCL, one cannot be sure how much test item remained in the solution to react with the peptides. Consequently, this negative result is uncertain and should be interpreted with due care.

3. INTRODUCTION

The objective of this study was to determine the reactivity of towards model synthetic peptides containing either cysteine or lysine, and to categorize the test item in one of four classes of reactivity for supporting the discrimination between skin sensitizers and non-sensitizers.

Background

The Direct Peptide Reactivity Assay (DPRA) is an in chemico method which quantifies the remaining concentration of cysteine- or lysine-containing peptide following 24 hours incubation with the test item at 25°C. The synthetic peptides contain phenylalanine to aid in the detection. The relative peptide concentration is measured by high-performance liquid chromatography (HPLC) with gradient elution and photodiode array (PDA) detection at 220 nm and 258 nm. Cysteine and lysine peptide Percent Depletion Values are calculated and used in a prediction model which allows assigning the test item to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

The design of this study is based on the following study guideline:

- OECD Guideline for the Testing of Chemicals, Guideline 442C. *In Chemico Skin Sensitization: Direct Peptide Reactivity Assay (DPRA)* (4 February 2015)

The Study Director signed the study plan on 29 Mar 2018. The experimental start date was 04 Apr 2018, and the experimental completion date was 18 Apr 2018.

The study plan and study plan deviation are presented in Appendix 5.

4. MATERIALS AND METHODS

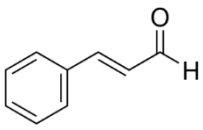
4.1. Test Item and Reference Item

4.1.1. Test Item (

Identification:

Appearance:	Clear colourless liquid
Batch:	12639
Purity/Composition:	See Certificate of Analysis
Test item storage:	At room temperature container flushed with nitrogen
Stable under storage conditions until:	08 November 2020 (expiry date)
Additional information	
Test Facility Test Item Number:	209150/A
Purity/Composition correction factor:	No correction factor required
Test item handling:	Handle in glove box (nitrogen environment)
Stability at higher temperatures:	Yes, maximum temperature: 100°C, maximum duration: 60 minutes
Chemical name (IUPAC, synonym or trade name:	
CAS number:	

4.1.2. Reference Item (Positive Control Cinnamic Aldehyde)

Identification:	Cinnamic aldehyde
Test Facility Test Item Number:	RS473/A
Appearance:	Yellow liquid
CAS Number:	104-55-2
Molecular Structure:	
Molecular Formula:	C_9H_8O
Molecular Weight:	132.16 g/mol
Batch:	MKBP1014V
Purity:	98.4%
Test item storage:	In the refrigerator (2-8°C)

Stable under storage conditions until:	31 May 2018
Supplier:	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Purity/composition correction factor:	Yes

For Certificate of Analysis see Appendix 6.

4.2. Test Item Characterization

The Sponsor provided to the Test Facility documentation of the identity, purity, composition and stability for the test item. A Certificate of Analysis was provided to the Test Facility and is presented in Appendix 6.

4.3. Reserve Samples

For each batch (lot) of test item, a reserve sample (about 0.5 gram) was collected and maintained under the appropriate storage conditions by the Test Facility and destroyed after the expiration date.

4.4. Test Item Inventory and Disposition

Records of the receipt, distribution, and storage of test item were maintained. With the exception of reserve samples, all unused Sponsor-supplied test item will be discarded after completion of the scheduled program of work. Records of the decisions made will be kept at the Test Facility.

4.5. Dose Formulation and Analysis

4.5.1. Preparation of Test Item

No correction for the purity/composition of the test item was performed.

Solubility of the test item in an appropriate solvent was assessed before performing the DPRA. An appropriate solvent dissolved the test item completely, i.e. by visual inspection the solution had to be not cloudy nor have noticeable precipitate. The following solvents were evaluated: acetonitrile (ACN), Milli-Q water (MQ), ACN:MQ (1:1, v/v), isopropanol, acetone:ACN (1:1, v/v) and dimethylsulfoxide (DMSO):ACN (1:9, v/v).

Test item stock solutions were prepared freshly for each reactivity assay.

For both the cysteine and lysine reactivity assay 63.85 mg of test item was pre-weighed into a clean amber glass vial and dissolved, just before use, in 1835 µL isopropanol after vortex mixing to obtain a 100 mM solution. Visual inspection of the forming of a clear solution was considered sufficient to ascertain that the test item was dissolved. The test item, positive control and peptide samples were prepared less than 4 hours before starting the incubation of the cysteine (cys) or lysine (lys) reactivity assay, respectively.

Any residual volumes were discarded.

4.6. Test System

Test system	Synthetic peptides containing cysteine (SPCC) (Ac-RFAA**C**AA-COOH) or synthetic peptides containing lysine (SPCL) (Ac-RFAA**K**AA-COOH). The molecular weight is 750.9 g/mol for SPCC and 775.9 g/mol for SPCL.

Rationale	Recommended test system in the international OECD guideline for DPRA studies.
Source	JPT Peptide Technologies GmbH, Berlin, Germany.
Batch	See Appendix 7 for detailed information.
Storage	The peptides were stored in the freezer (≤-15°C) for a maximum of 6 months.

4.7. Reagents

Acetone	HiPersolv, VWR international, Amsterdam, The Netherlands
Acetonitrile (ACN)	HPLC grade, Fisher Chemicals, Loughborough, England
Ammonium acetate	Fractopur, Merck, Darmstadt, Germany
Ammonium hydroxide	25%, Merck
Dimethylsulfoxide (DMSO)	Seccosolv, Merck
Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$)	Emsure, Merck
Isopropanol	LiChrosolv, Merck
MilliQ-water (MQ)	Tap water purified by reversed osmosis and subsequently passed over activated carbon and ion exchange cartridges; Millipore, Bedford, MA, USA
Sodium dihydrogenphosphate dehydrate ($NaH_2PO_4 \cdot H_2O$)	Emsure, Merck
Trifluoroacetic acid (TFA)	>99%, Sigma Aldrich, Zwijndrecht, The Netherlands

4.8. Experimental Design

4.8.1. Preparation of Solutions for Cysteine Reactivity Assay

4.8.1.1. Synthetic Peptide Containing Cysteine (SPCC) Stock Solution

A stock solution of 0.667 mM SPCC (0.501 mg SPCC/mL) was prepared by dissolving 10 mg of SPCC in 19.96 mL phosphate buffer pH 7.5. The mixture was stirred for 5 minutes followed by 5 minutes sonication.

4.8.1.2. SPCC Reference Control Solutions

Three 0.5 mM SPCC reference control (RC) solutions (RCcysA, RCcysB and RCcysC) were prepared in amber vials by mixing 750 µL of the 0.667 mM SPCC stock solution with 250 µL ACN. In addition, a $RCcysC_{isopropanol}$ sample was included to evaluate the effect of the solvent that was used to dissolve the test item on the Percent Peptide Depletion. The $RCcysC_{isopropanol}$ sample was prepared by mixing 750 µL of the 0.667 mM SPCC stock solution with 200 µL ACN and 50 µL isopropanol.

4.8.1.3. SPCC Calibration Curve

A SPCC calibration curve was prepared as described in the table below.

Preparation of SPCC Calibration Curve

SPCC calibration solutions	SPCC concentration (mM)	Preparation
STDcys1	0.534	1600 µL stock solution of 0.667 mM SPCC + 400 µL ACN
STDcys2	0.267	1 mL STDcys1 + 1 mL STDcys7
STDcys3	0.133	1 mL STDcys2 + 1 mL STDcys7
STDcys4	0.067	1 mL STDcys3 + 1 mL STDcys7
STDcys5	0.033	1 mL STDcys4 + 1 mL STDcys7
STDcys6	0.017	1 mL STDcys5 + 1 mL STDcys7
STDcys7	0	8 mL phosphate buffer (pH 7.5) + 2 mL ACN

4.8.1.4. Co-elution Control, Test Item and Positive Control Samples

The co-elution control (CC) samples, test item samples and the cinnamic aldehyde positive control samples (PC) were prepared as described in the table below.

Preparation of Co-elution Control, Test Item and Positive Control Samples

Sample	Replicates	Sample code	Preparation
Co-elution control (CC)	1	CCcys-209150/A	750 µL Phosphate buffer pH 7.5 200 µL ACN 50 µL 209150/A test solution (100 mM)
Cinnamic aldehyde (PC)	3	PCcys-1 to PCcys-3	750 µL Stock solution of 0.667 mM SPCC 200 µL ACN 50 µL Cinnamic aldehyde solution (100 mM in ACN)
Test item 209150/A	3	209150/A-cys-1 to 209150/A-cys-3	750 µL Stock solution of 0.667 mM SPCC 200 µL ACN 50 µL 209150/A test solution (100 mM)

4.8.2. Preparation of Solutions for Lysine Reactivity Assay

4.8.2.1. Synthetic Peptide Containing Lysine (SPCL) Stock Solution

A stock solution of 0.667 mM SPCL (0.518 mg SPCL/mL) was prepared by dissolving 10 mg of SPCL in 19.31 mL of ammonium acetate buffer pH 10.2 followed by stirring for 5 minutes.

4.8.2.2. SPCL Reference Control Solutions

Three 0.5 mM SPCL reference control (RC) solutions (RClysA, RClysB and RClysC) were prepared in amber vials by mixing 750 µL of the 0.667 mM SPCL stock solution with 250 µL ACN. In addition, a $RClysC_{isopropanol}$ sample was included to evaluate the effect of the solvent that was used to dissolve the test item on the Percent Peptide Depletion. The $RClysC_{isopropanol}$ sample was prepared by mixing 750 µL of the 0.667 mM SPCL stock solution with 250 µL isopropanol.

4.8.2.3. SPCL Calibration Curve

A SPCL peptide calibration curve was prepared as described in the table below.

Preparation of SPCL Calibration Curve

SPCL calibration solutions	SPCL concentration (mM)	Preparation
STDlys1	0.534	1600 µL stock solution of 0.667 mM SPCL + 400 µL ACN
STDlys2	0.267	1 mL STDlys1 + 1 mL STDlys7
STDlys3	0.133	1 mL STDlys2 + 1 mL STDlys7
STDlys4	0.067	1 mL STDlys3 + 1 mL STDlys7
STDlys5	0.033	1 mL STDlys4 + 1 mL STDlys7
STDlys6	0.017	1 mL STDlys5 + 1 mL STDlys7
STDlys7	0	8 mL ammonium acetate buffer (pH 10.2) + 2 mL ACN

4.8.2.4. Co-elution Control, Test Item and Positive Control Samples

The co-elution control (CC) samples, test item samples and the cinnamic aldehyde positive control samples (PC) were prepared as described in the table below.

Preparation of Co-elution Control, Test Item and Positive Control Samples

Sample	Replicates	Sample code	Preparation
Co-elution control (CC)	1	CClys-209150/A	750 µL Ammonium acetate buffer pH 10.2 250 µL 209150/A test solution (100 mM)
Cinnamic aldehyde (PC)	3	PClys-1 to PClys-3	750 µL Stock solution of 0.667 mM SPCL 250 µL Cinnamic aldehyde solution (100 mM in ACN)
Test item 209150/A	3	209150/A-lys-1 to 209150/A-lys-3	750 µL Stock solution of 0.667 mM SPCL 250 µL 209150/A test solution (100 mM)

4.8.3. Sample Incubations

After preparation, the samples (reference controls, calibration solutions, co-elution control, positive controls and test item samples) were placed in the autosampler in the dark and incubated at 25±2.5°C. The incubation time between placement of the samples in the autosampler and analysis of the first RCcysB- or RClysB-sample was 23 hours. The time between the first RCcysB- or RClysB-injection and the last injection of a cysteine or lysine sequence, respectively, did not exceed 30 hours.

Prior to HPLC-PDA analysis the samples were visually inspected for precipitation.

4.8.4. HPLC-PDA Analysis

SPCC and SPCL peak areas in the samples were measured by HPLC-PDA. Sample analysis was performed using the following systems:

System 1 (used for Cysteine Reactivity Assay):

- Surveyor MS HPLC pump (Thermo Scientific, Breda, The Netherlands)
- MPS 3C autosampler (DaVinci, Rotterdam, The Netherlands)
- LC Column oven 300 (Thermo Scientific)
- Surveyor PDA detector (Thermo Scientific)

System 2 (used for Lysine Reactivity Assay):

- Surveyor MS HPLC pump (Thermo Scientific, Breda, The Netherlands)
- HTC PAL autosampler (DaVinci, Rotterdam, The Netherlands)
- Column Oven #151006 (Grace, Worms, Germany)
- Surveyor PDA detector (Thermo Scientific)

All samples were analyzed according to the HPLC-PDA method presented in Table 1 (Appendix 1). The HPLC sequences of the cysteine and lysine reactivity assay for the test item are presented in Table 2 (Appendix 1).

5. ACCEPTABILITY CRITERIA

The following criteria had to be met for a run to be considered valid:

a) The standard calibration curve had to have an r^2>0.99.

b) The mean Percent Peptide Depletion value of the three replicates for the positive control cinnamic aldehyde had to be between 60.8% and 100% for SPCC and between 40.2% and 69.0% for SPCL.

c) The maximum standard deviation (SD) for the positive control replicates had to be <14.9% for the Percent Cysteine Peptide Depletion and <11.6% for the Percent Lysine Peptide Depletion.

d) The mean peptide concentration of Reference Controls A had to be 0.50 ± 0.05 mM.

e) The Coefficient of Variation (CV) of peptide areas for the nine Reference Controls B and C in ACN had to be <15.0%.

The following criteria had to be met for a test item's results to be considered valid:

a) The maximum SD for the test item replicates had to be <14.9% for the Percent Cysteine Depletion and <11.6% for the Percent Lysine Depletion.

b) The mean peptide concentration of the three Reference Controls C in the appropriate solvent had to be 0.50±0.05 mM.

6. ANALYSIS

6.1. Data Evaluation

The concentration of SPCC or SPCL was photometrically determined at 220 nm in each sample by measuring the peak area of the appropriate peaks by peak integration and by calculating the concentration of peptide using the linear calibration curve derived from the standards.

The Percent Peptide Depletion was determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant reference controls C according to the following formula:

$$\text{Percent Peptide Depletion} = \left[1 - \left(\frac{\text{Peptide Peak Area in Replicate Injection (at 220 nm)}}{\text{Mean Peptide Peak Area in Reference Controls (at 220 nm)}}\right)\right] \times 100$$

In addition, the absorbance at 258 nm was determined in each sample by measuring the peak area of the appropriate peaks by peak integration. The ratio of the 220 nm peak area and the 258 nm peak was used as an indicator of co-elution. For each sample, a ratio in the range of 90%<mean area ratio of control samples<110% gives a good indication that co-elution has not occurred.

6.2. Data Interpretation

The mean Percent Cysteine Depletion and Percent Lysine Depletion were calculated for the test item. Negative depletion was considered as "0" when calculating the mean. By using the Cysteine 1:10 / Lysine 1:50 prediction model (see table below), the threshold of 6.38% average peptide depletion was used to support the discrimination between a skin sensitizer and a non-sensitizer.

Cysteine 1:10 / Lysine 1:50 Prediction Model

Mean of cysteine and lysine % depletion	Reactivity class	DPRA prediction
0% ≤ Mean % depletion ≤ 6.38%	No or minimal reactivity	Negative
6.38% < Mean % depletion ≤ 22.62%	Low reactivity	Positive
22.62% < Mean % depletion ≤ 42.47%	Moderate reactivity	
42.47% < Mean % depletion ≤ 100%	High reactivity	

7. COMPUTERIZED SYSTEMS

Critical computerized systems used in the study are listed below. All computerized systems used in the conduct of this study have been validated; when a particular system has not satisfied all requirements, appropriate administrative and procedural controls were implemented to assure the quality and integrity of data.

Critical Computerized Systems

System name	Version No.	Description of Data Collected and/or Analyzed
REES Centron	SQL 2.0	Temperature, relative humidity and/or atmospheric pressure monitoring
Xcalibur	2.0	System control, data acquisition and integration

8. RETENTION OF RECORDS

All study-specific raw data, documentation, study plan, and final reports from this study were archived at the Test Facility by no later than the date of final report issue unless otherwise specified in the study plan. At least five years after issue of the final report, the Sponsor will be contacted. Electronic data generated by the Test Facility were archived as noted above.

9. RESULTS

9.1. Solubility Assessment of the Test Item

At a concentration of 110 mM, was not soluble in ACN, MQ, ACN:MQ (1:1, v/v) and DMSO:ACN (1:9, v/v), but was soluble in isopropanol and acetone:ACN (1:1, v/v). Since the use of isopropanol was preferred, this solvent was selected to dissolve the test item in this DPRA study.

9.2. Cysteine Reactivity Assay

The reactivity of towards SPCC was determined by quantification of the remaining concentration of SPCC using HPLC-PDA analysis, following 23 hours of incubation at 25±2.5°C. Representative chromatograms of CCcys-209150/A and 209150/A-cys samples are presented in Appendix 4. An overview of the retention time at 220 nm and peak areas at 220 nm and 258 nm are presented in Table 3 (Appendix 3).

9.2.1. Acceptability of the Cysteine Reactivity Assay

The SPCC standard calibration curve is presented in Figure 1 (Appendix 2). The correlation coefficient (r^2) of the SPCC standard calibration curve was 0.998. Since the r^2 was >0.99, the SPCC standard calibration curve was accepted.

The results of the Reference Control samples A, C and $C_{isopropanol}$ are presented in Table 4 (Appendix 3). The mean peptide concentration of Reference Controls A was 0.470 ± 0.020 mM, the mean peptide concentration of Reference Controls C was 0.491 ± 0.037 mM and the mean peptide concentration of Reference Controls $C_{isopropanol}$ was 0.506 ± 0.013 mM. The means of Reference Control samples A, C and $C_{isopropanol}$ were all within the acceptance criteria of 0.50 ± 0.05 mM. This confirms the suitability of the HPLC system and indicates that the solvent (isopropanol) used to dissolve the test item did not impact the Percent SPCC Depletion.

The SPCC peak areas for Reference controls B and C are presented in Table 5 (Appendix 3). The Coefficient of Variation (CV) of the peptide areas for the nine Reference Controls B and C was 5.0%. This was within the acceptance criteria (CV <15.0%) and confirms the stability of the HPLC run over time.

The SPCC A_{220}/A_{258} area ratios of Reference controls A, B and C are presented in Table 6 (Appendix 3). The mean area ratio (A_{220}/A_{258}) of the Reference Control samples was 19.18. The mean A_{220}/A_{258} ratio ± 10% range was 17.26-21.10. Each sample showing an A_{220}/A_{258} ratio within this range gives an indication that co-elution has not occurred.

The results of the positive control cinnamic aldehyde are presented in Table 7 (Appendix 3). The Percent SPCC Depletion was calculated versus the mean SPCC peak area of Reference Controls C. The mean Percent SPCC Depletion for the positive control cinnamic aldehyde was 73.6% ± 2.0%. This was within the acceptance range of 60.8% to 100% with a SD that was below the maximum (SD <14.9%).

9.2.2. Results Cysteine Reactivity Assay for the Test Item

Preparation of a 100 mM stock solution in isopropanol showed that the test item was dissolved completely. Upon preparation and after incubation, both the co-elution control (CC) as well as the test item samples were visually inspected. No precipitate was observed in any of the samples.

The results of the cysteine reactivity assay for the test item are presented in Table 8 (Appendix 3). In the CC sample no peak was observed at the retention time of SPCC (see

chromatogram in Appendix 4). This demonstrated that there was no co-elution of the test item with SPCC. For the 209150/A-cys samples, the mean SPCC A_{220}/A_{258} area ratio was 18.94. Since this was within the 17.26-21.10 range, this again indicated that there was no co-elution of the test item with SPCC.

The Percent SPCC Depletion was calculated versus the mean SPCC peak area of Reference Controls $C_{isopropanol}$. The mean Percent SPCC Depletion for the test item was 3.1% ± 5.3%.

9.3. Lysine Reactivity Assay

The reactivity of towards SPCL was determined by quantification of the remaining concentration of SPCL using HPLC-PDA analysis, following 23 hours of incubation at 25±2.5°C. Representative chromatograms of CClys-209150/A and 209150/A-lys samples are presented in Appendix 4. An overview of the retention time at 220 nm and peak areas at 220 nm and 258 nm are presented in Table 9 (Appendix 3).

9.3.1. Acceptability of the Lysine Reactivity Assay

The SPCL standard calibration curve is presented in Figure 2 (Appendix 2). The correlation coefficient (r^2) of the SPCL standard calibration curve was 0.998. Since the r^2 was >0.99, the SPCL standard calibration curve was accepted.

The results of the Reference Control samples A, C and $C_{isopropanol}$ are presented in Table 10 (Appendix 3). The mean peptide concentration of Reference Controls A was 0.485 ± 0.007 mM, the mean peptide concentration of Reference Controls C was 0.484 ± 0.003 mM and the mean peptide concentration of Reference Controls $C_{isopropanol}$ was 0.501 ± 0.004 mM. The means of Reference Control samples A, C and $C_{isopropanol}$ were all within the acceptance criteria of 0.50 ± 0.05 mM. This confirms the suitability of the HPLC system and indicates that the solvent (isopropanol) used to dissolve the test item did not impact the Percent SPCL Depletion.

The SPCL peak areas for Reference controls B and C are presented in Table 11 (Appendix 3). The CV of the peptide areas for the nine Reference Controls B and C was 1.0%. This was within the acceptance criteria (CV <15.0%) and confirms the stability of the HPLC run over time.

The SPCL A_{220}/A_{258} area ratios of Reference controls A, B and C are presented in Table 12 (Appendix 3). The mean area ratio (A_{220}/A_{258}) of the Reference Control samples was 15.34. The mean A_{220}/A_{258} ratio ± 10% range was 13.80-16.87. Each sample showing an A_{220}/A_{258} ratio within this range gives an indication that co-elution has not occurred.

The results of the positive control cinnamic aldehyde are presented in Table 13 (Appendix 3). The Percent SPCL Depletion was calculated versus the mean SPCL peak area of Reference Controls C. The mean Percent SPCL Depletion for the positive control cinnamic aldehyde was 55.0% ± 0.1%. This was within the acceptance range of 40.2% to 69.0% with a SD that was below the maximum (SD <11.6%).

9.3.2. Results Lysine Reactivity Assay for the Test Item

Preparation of a 100 mM stock solution in isopropanol showed that the test item was dissolved completely. Upon preparation and after incubation, both the CC as well as the test item samples were visually inspected. Upon preparation as well as after incubation a precipitate was observed in the CC and the test item samples. In this case one cannot be sure how much test item remained in the solution to react with the peptide.

The results of the lysine reactivity assay for the test item are presented in Table 14 (Appendix 3). In the CC sample no peak was observed at the retention time of SPCL (see chromatogram in Appendix 4). This demonstrated that there was no co-elution of the test item with SPCL. For the 209150/A-lys samples, the mean SPCL A_{220}/A_{258} area ratio was 15.80. Since this was within the 13.80-16.87 range, this again indicated that there was no co-elution of the test item with SPCL.

The Percent SPCL Depletion was calculated versus the mean SPCL peak area of Reference Controls $C_{isopropanol}$. The mean Percent SPCL Depletion for the Test Item was 0.3% ± 0.4%.

9.4. DPRA Prediction and Reactivity Classification

Upon preparation as well as after incubation of the SPCC test item samples, no precipitate was observed in any of the samples. However, upon preparation as well as after incubation of the SPCL test item samples, a precipitate was observed.

An overview of the individual results of the cysteine and lysine reactivity assays as well as the mean of the SPCC and SPCL depletion are presented in the table below. In the cysteine reactivity assay the test item showed 3.1% SPCC depletion while in the lysine reactivity assay the test item showed 0.3% SPCL depletion. The mean of the SPCC and SPCL depletion was 1.7% and as a result the test item was negative in the DPRA and was classified in the "no or minimal reactivity class" when using the Cysteine 1:10 / Lysine 1:50 prediction model.

SPCC and SPCL Depletion, DPRA Prediction and Reactivity Classification for the Test Item

Test item	SPCC depletion		SPCL depletion		Mean of SPCC and SPCL depletion	DPRA prediction and reactivity classification
	Mean	± SD	Mean	± SD		Cysteine 1:10 / Lysine 1:50 prediction model
	3.1%	±5.3%	0.3%	±0.4%	1.7%	Negative: No or minimal reactivity

SD = Standard Deviation.

10. CONCLUSION

In conclusion, this DPRA test is valid. was negative in the DPRA and was classified in the "no or minimal reactivity class" when using the Cysteine 1:10 / Lysine 1:50 prediction model. However, since precipitation was observed upon preparation and after the incubation period for SPCL, one cannot be sure how much test item remained in the solution to react with the peptides. Consequently, this negative result is uncertain and should be interpreted with due care.

11. LIST OF ABBREVIATIONS

ACN	Acetonitrile
CC	Co-elution Control
CV	Coefficient of Variation
Cys	Cysteine
DMSO	Dimethylsulphoxide
DPRA	Direct Peptide Reactivity Assay
GLP	Good Laboratory Practice
HPLC	High-Performance Liquid Chromatography
Lys	Lysine
MQ	Milli-Q water
OECD	Organization for Economic Cooperation and Development
PC	Positive Control
PDA	Photodiode Array
QA	Quality Assurance department
RC	Reference Control
SD	Standard Deviation
SPCC	Synthetic Peptide Containing Cysteine
SPCL	Synthetic Peptide Containing Lysine
STD	Standard calibration solution
TFA	Trifluoroacetic acid

Appendix 1
Analytical Methods and HPLC Sequences

Table 1
HPLC-PDA Method for Determination of SPCC and SPCL

Mobile phase	A: 0.1% (v/v) TFA in Milli-Q water B: 0.085% (v/v) TFA in ACN	
Gradient	Cysteine	Lysine
	0 min 10% B 10 min 25% B 11 min 90% B 13 min 90% B 13.5 min 10% B 20 min 10% B	0 min 10% B 10 min 20% B 11 min 90% B 13 min 90% B 13.5 min 10% B 20 min 10% B
Flow	0.35 mL/min	
Injection volume	3 µL	
Sample tray temperature	Set at 25°C	
Column	Zorbax SB-C_{18}, 100 mm x 2.1 mm, df = 3.5 µm (Agilent Technologies, Santa Clara, CA, USA)	
Guard column	SecurityGuard™ cartridge for C_{18}, 4 x 2.0 mm (Phenomenex, Torrance, CA, USA)	
Column temperature	Set at 30°C	
Detection	Photodiode array detection, monitoring at 220 and 258 nm	

Table 2
Analysis Sequences of Cysteine and Lysine Reactivity Assays

Sample description	Cysteine reactivity assay	Lysine reactivity assay
Calibration standards (STD) and reference controls (RC)	STDcys7 STDcys6 STDcys5 STDcys4 STDcys3 STDcys2 STDcys1 Dilution buffer RCcysA-1 RCcysA-2 RCcysA-3	STDlys7 STDlys6 STDlys5 STDlys4 STDlys3 STDlys2 STDlys1 Dilution buffer RClysA-1 RClysA-2 RClysA-3
Co-elution controls (CC)	CCcys-209150/A	CClys-209150/A
Reference controls (RC)	RCcysB-1 RCcysB-2 RCcysB-3	RClysB-1 RClysB-2 RClysB-3
First set of replicates	RCcysC-1 RCcys$C_{isopropanol}$-1 PCcys-1 209150/A-cys-1	RClysC-1 RClys$C_{isopropanol}$-1 PClys-1 209150/A-lys-1
Second set of replicates	RCcysC-2 RCcys$C_{isopropanol}$-2 PCcys-2 209150/A-cys-2	RClysC-2 RClys$C_{isopropanol}$-2 PClys-2 209150/A-lys-2
Third set of replicates	RCcysC-3 RCcys$C_{isopropanol}$-3 PCcys-3 209150/A-cys-3	RClysC-3 RClys$C_{isopropanol}$-3 PClys-3 209150/A-lys-3
Reference controls	RCcysB-4 RCcysB-5 RCcysB-6	RClysB-4 RClysB-5 RClysB-6

STD = standard; RC = reference control; CC = co-elution control; PC = positive control.

Appendix 2
Figures

Figure 1
SPCC Calibration Curve

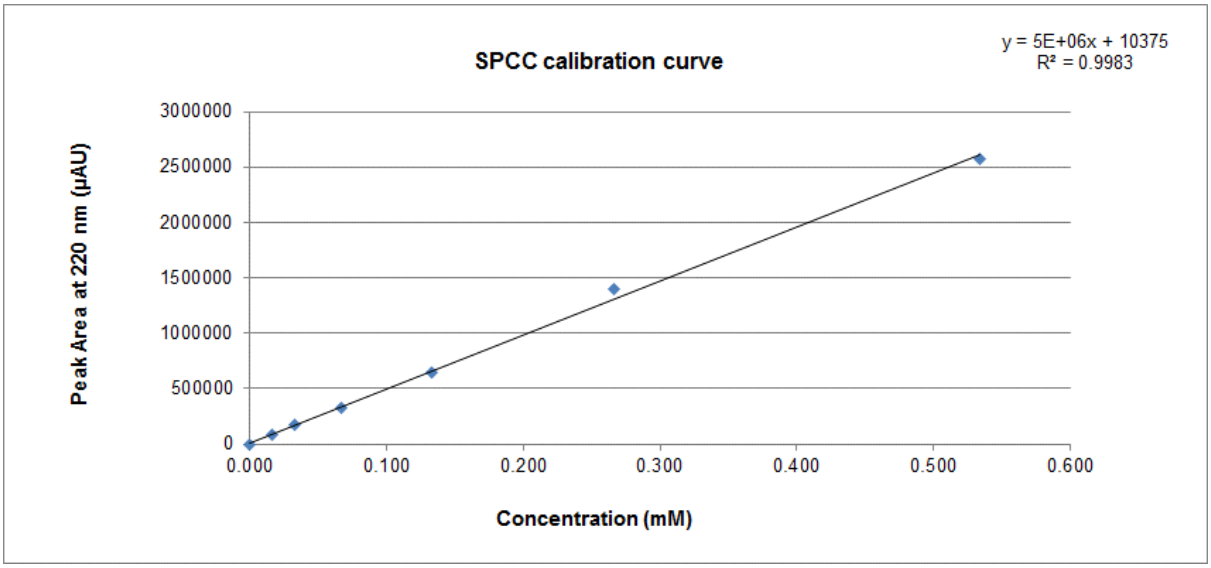
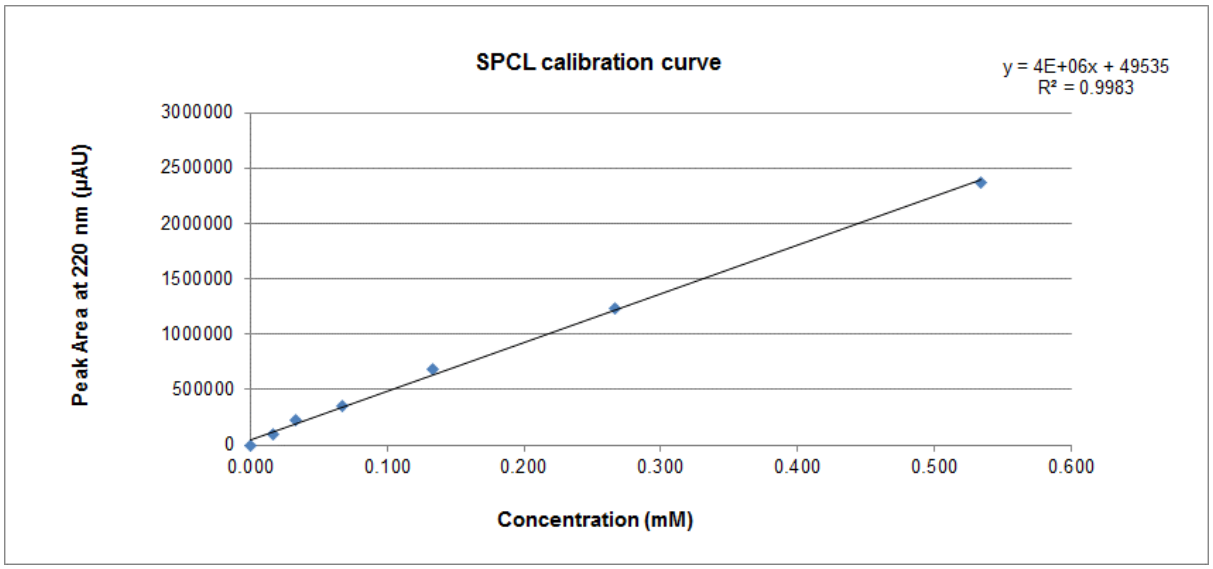


Figure 2
SPCL Calibration Curve



Appendix 3
Result Tables

Table 3
SPCC Retention Times and Peak Areas (at 220 nm and 258 nm)

Sample code	Retention time of peak at 220 nm (min)	Peak area at 220 nm (µAU)	Peak area at 258 nm (µAU)
STDcys7	NF	NF	NF
STDcys6	7.62	80166	3767
STDcys5	7.88	170004	7360
STDcys4	7.88	321233	15962
STDcys3	7.75	651278	31836
STDcys2	7.58	1392771	68451
STDcys1	7.53	2569217	137138
Dilution buffer	NF	NF	NF
RCcysA-1	7.88	2391110	123488
RCcysA-2	7.78	2300959	117861
RCcysA-3	7.57	2196884	114943
CCcys-209150/A	NF	NF	NF
RCcysB-1	7.83	2373223	122807
RCcysB-2	7.70	2391662	125888
RCcysB-3	7.58	2374916	123151
RCcysC-1	7.85	2400613	123315
$RCcysC_{isopropanol}$-1	7.75	2428072	127209
PCcys-1	7.58	583905	30552
209150/A-cys-1	7.57	2246469	117555
RCcysC-2	7.48	2217328	115447
$RCcysC_{isopropanol}$-2	7.58	2447044	128900
PCcys-2	7.85	639159	33916
209150/A-cys-2	7.72	2546084	134280
RCcysC-3	7.77	2581044	134746
$RCcysC_{isopropanol}$-3	7.90	2549569	136810
PCcys-3	7.82	677968	33367
209150/A-cys-3	7.70	2524494	134658
RCcysB-4	7.55	2545344	134861
RCcysB-5	7.53	2520047	133989
RCcysB-6	7.58	2581113	135707

NF = No peak found.

Table 4
SPCC Peak Area at 220 nm and Concentration in Reference Controls A, C and $C_{isopropanol}$

Sample code	Peak area at 220 nm (µAU)	Concentration (mM)
RCcysA-1	2391110	0.489
RCcysA-2	2300959	0.471
RCcysA-3	2196884	0.449
	Mean	0.470
	SD	0.020
Sample code	Peak area at 220 nm (µAU)	Concentration (mM)
RCcysC-1	2400613	0.491
RCcysC-2	2217328	0.454
RCcysC-3	2581044	0.528
	Mean	0.491
	SD	0.037
Sample code	Peak area at 220 nm (µAU)	Concentration (mM)
$RCcysC_{isopropanol}$-1	2428072	0.497
$RCcysC_{isopropanol}$-2	2447044	0.501
$RCcysC_{isopropanol}$-3	2549569	0.522
	Mean	0.506
	SD	0.013

SD = Standard Deviation.

Table 5
SPCC Peak Area of Reference Controls B and C

Reference Controls in ACN	Peak area at 220 nm (µAU)
RCcysB-1	2373223
RCcysB-2	2391662
RCcysB-3	2374916
RCcysB-4	2545344
RCcysB-5	2520047
RCcysB-6	2581113
RCcysC-1	2400613
RCcysC-2	2217328
RCcysC-3	2581044
Mean	2442810
SD	122171
CV	5.0%

SD = Standard Deviation; CV = Coefficient of Variation.

Table 6
SPCC Area Ratio (A_{220}/A_{258}) of Reference Controls A, B and C

Reference Controls in ACN	Peak area at 220 nm (µAU)	Peak area at 258 nm (µAU)	Area ratio (A_{220}/A_{258})
RCcysA-1	2391110	123488	19.36
RCcysA-2	2300959	117861	19.52
RCcysA-3	2196884	114943	19.11
RCcysB-1	2373223	122807	19.32
RCcysB-2	2391662	125888	19.00
RCcysB-3	2374916	123151	19.28
RCcysB-4	2545344	134861	18.87
RCcysB-5	2520047	133989	18.81
RCcysB-6	2581113	135707	19.02
RCcysC-1	2400613	123315	19.47
RCcysC-2	2217328	115447	19.21
RCcysC-3	2581044	134746	19.15
		Mean	19.18

Table 7
SPCC Peak Area, Concentration and Depletion of the Cinnamic Aldehyde Positive Control Samples

Sample code	Peak area at 220 nm (µAU)	Concentration (mM)	SPCC Depletion
PCcys-1	583905	0.118	75.7%
PCcys-2	639159	0.129	73.4%
PCcys-3	677968	0.137	71.7%
		Mean	73.6%
		SD	2.0%

SD = Standard Deviation.

Table 8
SPCC Peak Area, Concentration, Depletion and Area Ratio (A_{220}/A_{258}) of the Test Item Samples

Sample code	Peak area at 220 nm (µAU)	Concentration (mM)	SPCC Depletion	Peak area at 258 nm (µAU)	Area ratio (A_{220}/A_{258})
209150/A-cys-1	2246469	0.460	9.2%	117555	19.11
209150/A-cys-2	2546084	0.521	0.0%	134280	18.96
209150/A-cys-3	2524494	0.517	0.0%	134658	18.75
		Mean	3.1%	NA	18.94
		SD	5.3%	NA	0.18

SD = Standard Deviation, NA = Not Applicable.

Table 9
SPCL Retention Times and Peak Areas (at 220 nm and 258 nm)

Sample code	Retention time of peak at 220 nm (min)	Peak area at 220 nm (µAU)	Peak area at 258 nm (µAU)
STDlys7	NF	NF	NF
STDlys6	6.43	92976	5106
STDlys5	6.45	227930	9959
STDlys4	6.40	348581	18949
STDlys3	6.47	683680	38129
STDlys2	6.40	1231836	76578
STDlys1	6.38	2370420	152317
Dilution buffer	NF	NF	NF
RClysA-1	6.40	2167298	140074
RClysA-2	6.42	2210338	142121
RClysA-3	6.42	2151583	141859
CClys-209150/A	NF	NF	NF
RClysB-1	6.40	2222707	142638
RClysB-2	6.38	2195019	141878
RClysB-3	6.38	2210726	142853
RClysC-1	6.40	2164959	146589
$RClysC_{isopropanol}$-1	6.35	2267001	145998
PClys-1	6.45	978757	69292
209150/A-lys-1	6.38	2231095	143255
RClysC-2	6.33	2168201	142923
$RClysC_{isopropanol}$-2	6.37	2249929	144209
PClys-2	6.43	975986	69218
209150/A-lys-2	6.40	2262835	143893
RClysC-3	6.40	2188719	140219
$RClysC_{isopropanol}$-3	6.33	2227876	143370
PClys-3	6.42	981703	75074
209150/A-lys-3	6.40	2291061	142222
RClysB-4	6.42	2173653	142918
RClysB-5	6.38	2191977	141874
RClysB-6	6.40	2152233	142611

NF = No peak found.

Table 10
SPCL Peak Area at 220 nm and Concentration in Reference Controls A, C and $C_{isopropanol}$

Sample code	Peak area at 220 nm (µAU)	Concentration (mM)
RClysA-1	2167298	0.483
RClysA-2	2210338	0.493
RClysA-3	2151583	0.479
	Mean	0.485
	SD	0.007
Sample code	Peak area at 220 nm (µAU)	Concentration (mM)
RClysC-1	2164959	0.482
RClysC-2	2168201	0.483
RClysC-3	2188719	0.488
	Mean	0.484
	SD	0.003
Sample code	Peak area at 220 nm (µAU)	Concentration (mM)
$RClysC_{isopropanol}$-1	2267001	0.505
$RClysC_{isopropanol}$-2	2249929	0.502
$RClysC_{isopropanol}$-3	2227876	0.497
	Mean	0.501
	SD	0.004

SD = Standard Deviation.

Table 11
SPCL Peak Area of Reference Controls B and C

Reference Controls in ACN	Peak area at 220 nm (µAU)
RClysB-1	2222707
RClysB-2	2195019
RClysB-3	2210726
RClysB-4	2173653
RClysB-5	2191977
RClysB-6	2152233
RClysC-1	2164959
RClysC-2	2168201
RClysC-3	2188719
Mean	2185355
SD	22726
CV	1.0%

SD = Standard Deviation; CV = Coefficient of Variation.

Table 12
SPCL Area Ratio (A_{220}/A_{258}) of Reference Controls A, B and C

Reference Controls in ACN	Peak area at 220 nm (µAU)	Peak area at 258 nm (µAU)	Area ratio (A_{220}/A_{258})
RClysA-1	2167298	140074	15.47
RClysA-2	2210338	142121	15.55
RClysA-3	2151583	141859	15.17
RClysB-1	2222707	142638	15.58
RClysB-2	2195019	141878	15.47
RClysB-3	2210726	142853	15.48
RClysB-4	2173653	142918	15.21
RClysB-5	2191977	141874	15.45
RClysB-6	2152233	142611	15.09
RClysC-1	2164959	146589	14.77
RClysC-2	2168201	142923	15.17
RClysC-3	2188719	140219	15.61
		Mean	15.34

Table 13
SPCL Peak Area, Concentration and Depletion of the Cinnamic Aldehyde Positive Control Samples

Sample code	Peak area at 220 nm (µAU)	Concentration (mM)	SPCL Depletion
PClys-1	978757	0.212	55.0%
PClys-2	975986	0.211	55.1%
PClys-3	981703	0.212	54.8%
		Mean	55.0%
		SD	0.1%

SD = Standard Deviation.

Table 14
SPCL Peak Area, Concentration, Depletion and Area Ratio (A_{220}/A_{258}) of the Test Item Samples

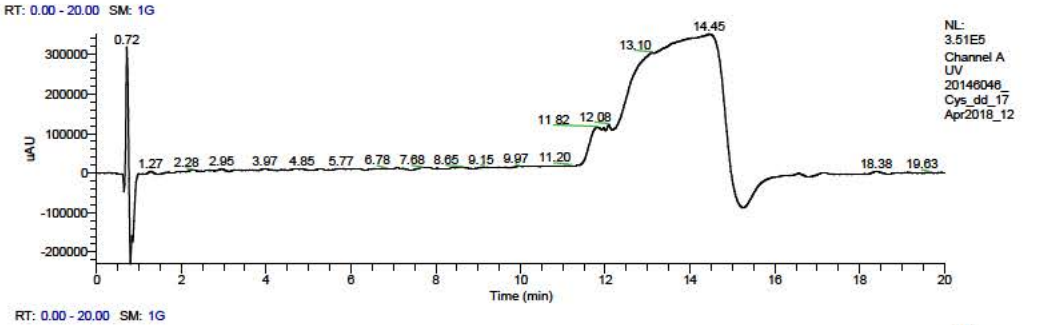
Sample code	Peak area at 220 nm (µAU)	Concentration (mM)	SPCL Depletion	Peak area at 258 nm (µAU)	Area ratio (A_{220}/A_{258})
209150/A-lys-1	2231095	0.497	0.8%	143255	15.57
209150/A-lys-2	2262835	0.505	0.0%	143893	15.73
209150/A-lys-3	2291061	0.511	0.0%	142222	16.11
		Mean	0.3%	NA	15.80
		SD	0.4%	NA	0.28

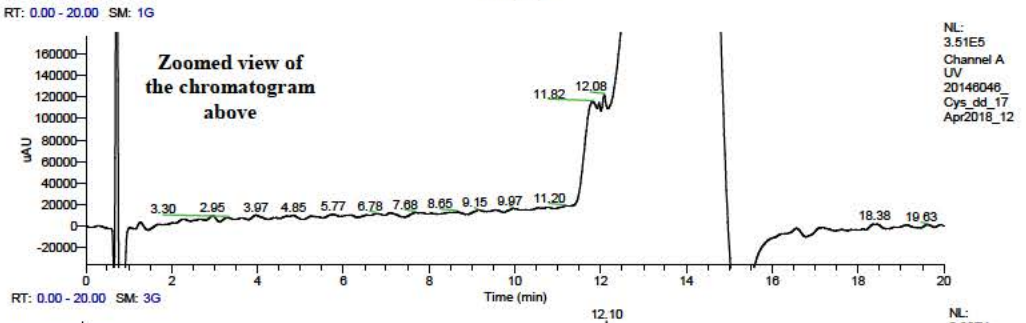
SD = Standard Deviation, NA = Not Applicable.

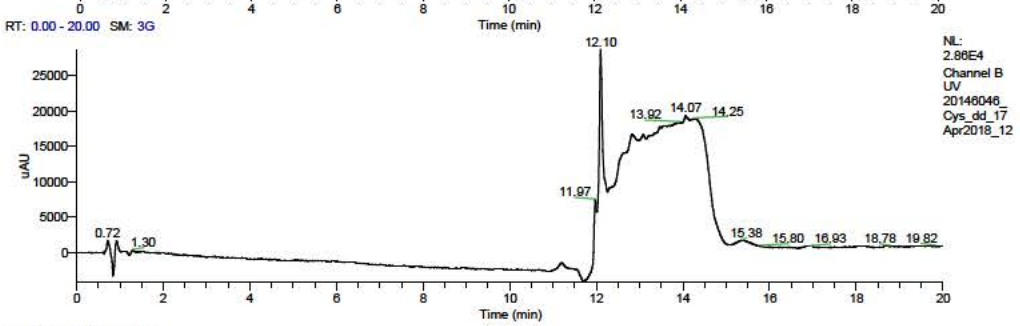
Appendix 4
HPLC Chromatograms

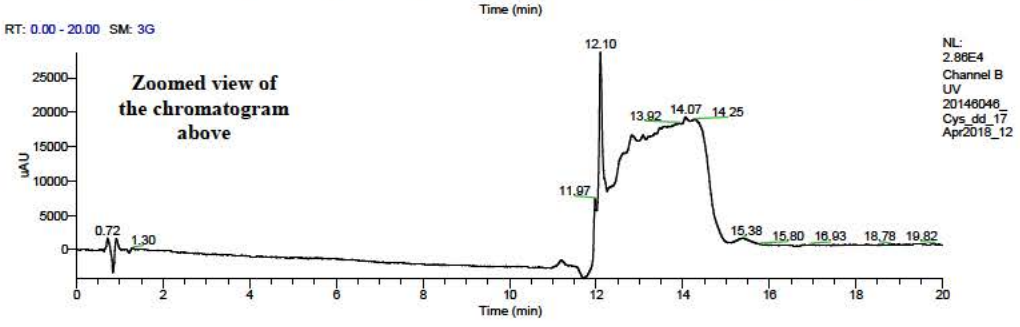
Chromatograms of the cysteine reactivity assay

Sample Name:	CCcys-209150/A	Channel A = 220 nm
Data File:	20146046_Cys_dd_17Apr2018_12	Channel B = 258 nm
Operator:	QMA	
Original Data Path:	D:\Projects\20146046\Cysteine 17Apr2018	
Acquisition Date:	04/18/18 11:05:57 AM	
Instrument Method:	D:\Projects\SOP methoden\DPRA\cysteine determination	
Injection Volume(µl):	3.00	
Run Time(min):	20.00	





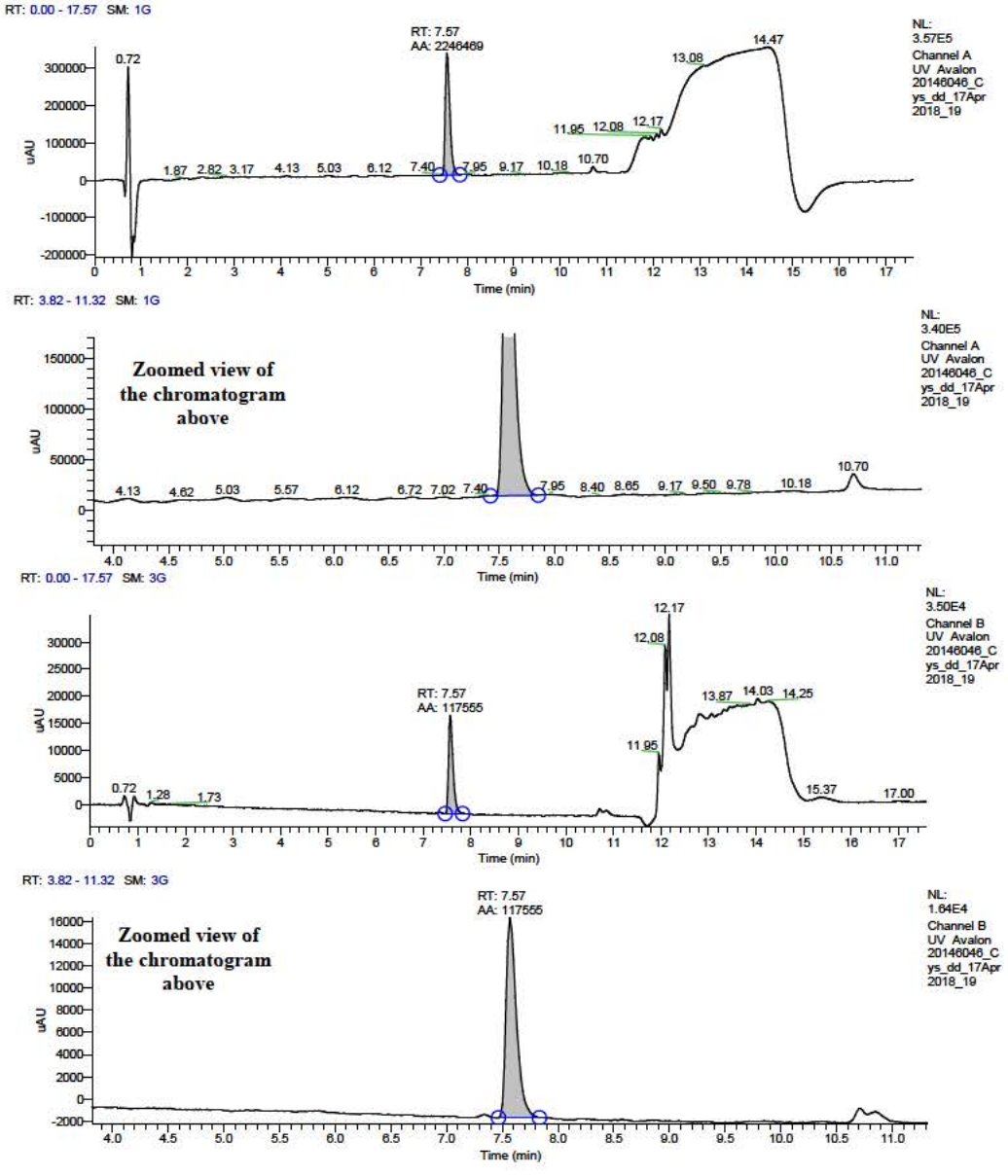




Component Name	Area	RT	Width	Height
Cysteine_258nm	N/A	N/A	N/A	N/A
Cysteine_220nm	N/A	N/A	N/A	N/A

Chromatograms of the cysteine reactivity assay *(continued)*

Sample Name:	209150/A-cys-1	Channel A = 220 nm
Data File:	20146046_Cys_dd_17Apr2018_19	Channel B = 258 nm
Operator:	QMA	
Original Data Path:	D:\Projects\20146046\Cysteine 17Apr2018	
Acquisition Date:	04/18/18 01:38:46 PM	
Instrument Method:	D:\Projects\SOP methoden\DPRA\cysteine determination	
Injection Volume(µl):	3.00	
Run Time(min):	20.00	

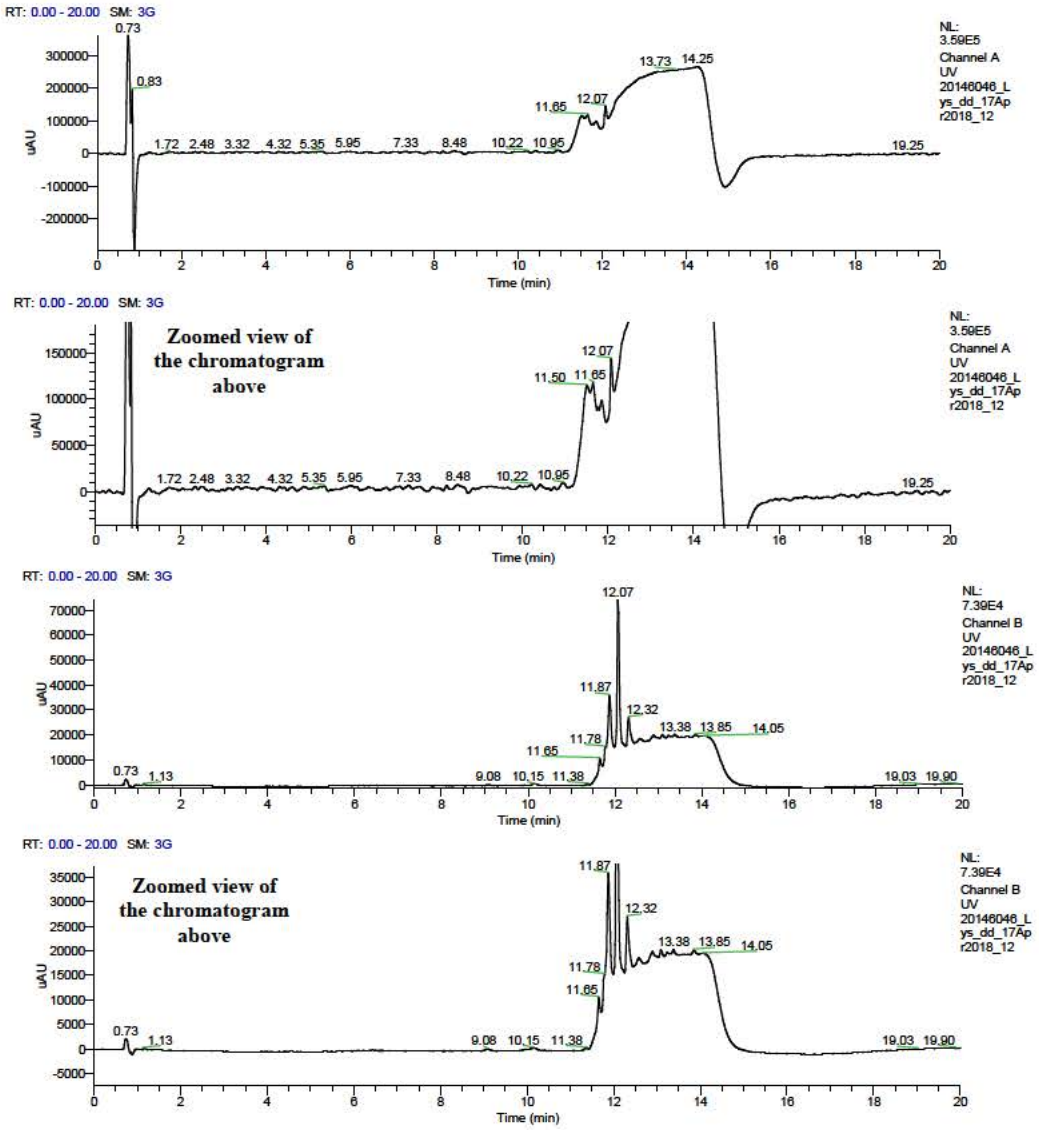


Component Name	Area	RT	Width	Height
Cysteine_220nm	2246469.50	7.57	0.43	325676.00
Cysteine_258nm	117554.75	7.57	0.37	18104.35

Chromatograms of the lysine reactivity assay

Sample Name:	CClys-209150/A
Data File:	20146046_Lys_dd_17Apr2018_12
Operator:	QMA
Original Data Path:	D:\Projects\20146046\Lysine 17Apr2018
Acquisition Date:	04/18/18 11:00:50 AM
Instrument Method:	D:\Projects\SOP methoden\DPRA\lysine determination
Injection Volume(µl):	3.00
Run Time(min):	20.00

Channel A = 220 nm
Channel B = 258 nm

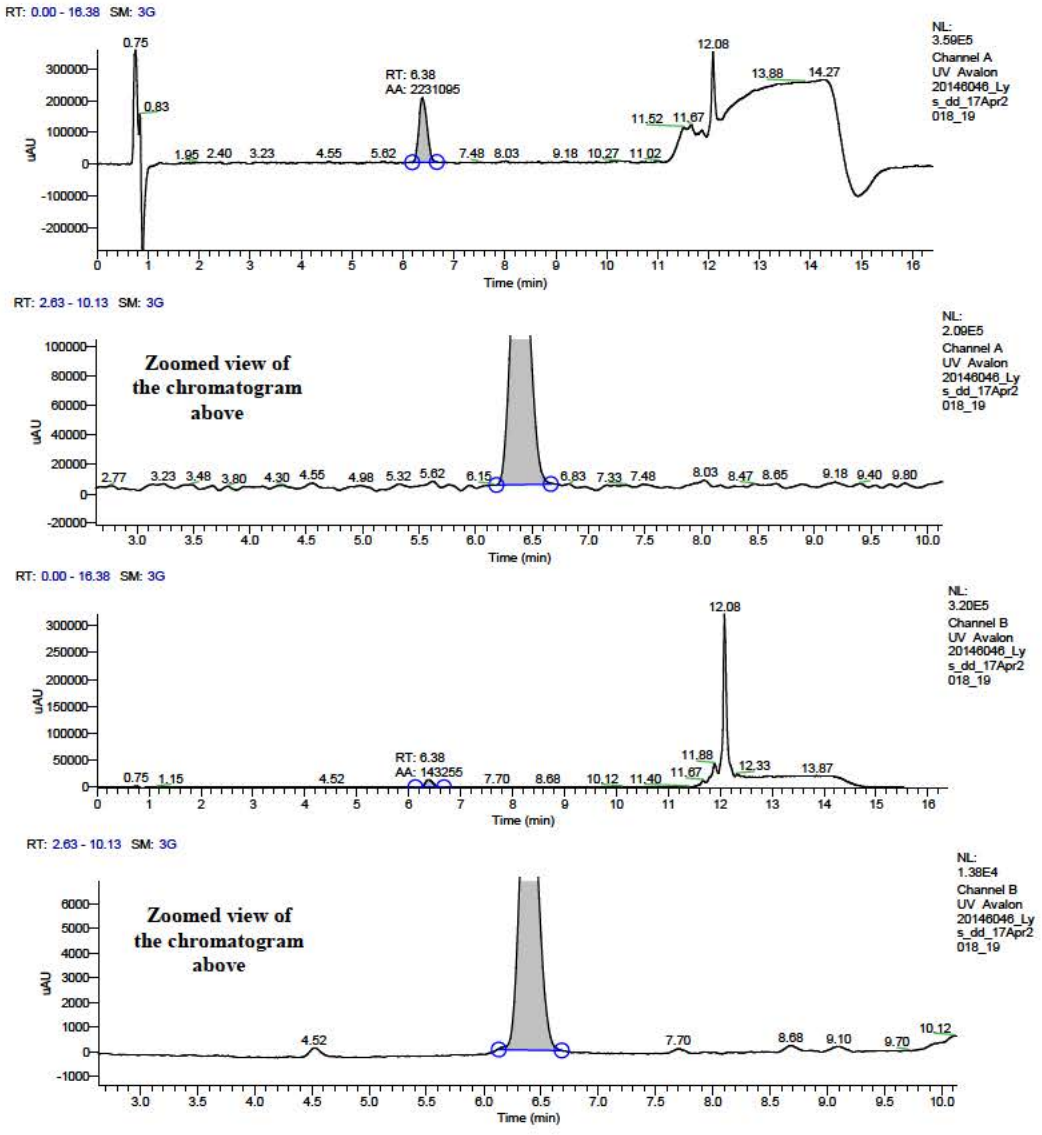


Component Name	Area	RT	Width	Height
Lysine_258nm	N/A	N/A	N/A	N/A
Lysine_220nm	N/A	N/A	N/A	N/A

Chromatograms of the lysine reactivity assay *(continued)*

Sample Name:	209150/A-lys-1
Data File:	20146046_Lys_dd_17Apr2018_19
Operator:	QMA
Original Data Path:	D:\Projects\20146046\Lysine 17Apr2018
Acquisition Date:	04/18/18 01:30:55 PM
Instrument Method:	D:\Projects\SOP methoden\DPRA\lysine determination
Injection Volume(µl):	3.00
Run Time(min):	20.00

Channel A = 220 nm
Channel B = 258 nm



Component Name	Area	RT	Width	Height
Lysine_220nm	2231094.86	6.38	0.48	202513.59
Lysine_258nm	143254.80	6.38	0.55	13739.94

Appendix 5
Study plan and Deviations

charles river

FINAL STUDY PLAN

Test Facility Study No. 20146046

Sponsor Reference No.

In Chemico Determination of the Skin Sensitization Potential of using the Direct Peptide Reactivity Assay (DPRA)

TEST FACILITY:
Charles River Laboratories Den Bosch BV
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

TABLE OF CONTENTS

1. OBJECTIVE(S)

The objective of this study is to determine the reactivity of
towards model synthetic peptides containing either cysteine or lysine, and to categorize the test item in one of four classes of reactivity for supporting the discrimination between skin sensitizers and non-sensitizers.

Background

The Direct Peptide Reactivity Assay (DPRA) is an in chemico method which quantifies the remaining concentration of cysteine- or lysine-containing peptide following 24 hours of incubation with the test item at 25°C. The synthetic peptides contain phenylalanine to aid in the detection. The relative peptide concentration is measured by high-performance liquid chromatography (HPLC) with gradient elution and photodiode array (PDA) detection at 220 nm and 258 nm. Cysteine and lysine peptide Percent Depletion Values are calculated and used in a prediction model which allows assigning the test item to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual applicable dates will be included in the Final Report.

Experimental Start Date:	02 Apr 2018 (First date of study-specific data collection)
Experimental Completion Date:	13 May 2018 (Last date data are collected from the study)
Unaudited Draft Report:	27 May 2018

3. GUIDELINES FOR STUDY DESIGN

The design of this study was based on the study objective(s), the overall product development strategy for the test item and the following study design guideline:

- OECD Guideline for the Testing of Chemicals, Guideline 442C. *In Chemico Skin Sensitization: Direct Peptide Reactivity Assay (DPRA)* (4 February 2015)

4. REGULATORY COMPLIANCE

The study will be performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America, Japan, and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

5. QUALITY ASSURANCE

5.1. Test Facility

The Test Facility Quality Assurance Unit (QAU) will monitor the study to assure the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the study plan, conduct study and/or process inspections at intervals adequate to assure the integrity of the study, and

audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

The Test Facility QAU contact is indicated below:

C.J. Mitchell, BSc
Address as cited for Test Facility
Tel: +31 73 640 6700
E-mail: christine.mitchell@crl.com

6. SPONSOR

7. RESPONSIBLE PERSONNEL

Study Director

J. Reinen, PhD.
Address as cited for Test Facility
Tel: +31 73 640 6700
E-mail: jelle.reinen@crl.com

Management Contact

E.J. van de Waart, MSc, ERT
Head of Discovery & Environmental Sciences
Address as cited for Test Facility
Tel: +31 73 640 6700
E-mail: beppy.vandewaart@crl.com

8. TEST ITEM AND REFERENCE ITEM

8.1. Test Item (Perfluoroalkyl Vinyl Ether (n=1))

8.1.1.

Appearance:	Clear colourless liquid
Batch:	12639
Purity/Composition:	See Certificate of Analysis
Test item storage:	At room temperature container flushed with nitrogen
Stable under storage conditions until:	08 November 2020 (expiry date)

For Certificate of Analysis see ATTACHMENT B.

Additional information

Test Facility test item number:	209150/A
Purity/Composition correction factor:	No correction factor required
Test item handling:	Handle in glove box (nitrogen environment)
Stability at higher temperatures:	Yes, maximum temperature: 100°C, maximum duration: 60 minutes

Solubility in vehicle:

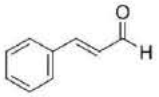
• Water	Not determined, but very low (the substance can be washed with water during purification and forms a lower organic layer)
• Hexane	Miscible
• Acetone	0.12 g/mL

Stability in vehicle:

• Water	Stable
• Hexane	Stable
• Acetone	Stable

General information:

8.2. Reference Item (Positive Control Cinnamic Aldehyde)

Identification:	Cinnamic aldehyde
Test Facility Test Item Number:	RS473/A
Appearance:	Yellow liquid
CAS Number:	104-55-2
Molecular Structure:	
Molecular Formula:	C_9H_8O
Molecular Weight:	132.16 g/mol

Batch:	MKBP1014V
Purity:	98.4%
Test item storage:	In the refrigerator (2-8°C)
Stable under storage conditions until:	31 May 2018
Supplier:	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Purity/composition correction factor:	Yes

8.3. Test Item Characterization

The Sponsor will provide to the Test Facility documentation of the identity, purity, composition, and stability for the test item. If available, a Certificate of Analysis or equivalent documentation will be provided for inclusion in the Final Report. The Sponsor will also provide information concerning the regulatory standard that was followed for these evaluations.

The Sponsor has appropriate documentation on file concerning the method of synthesis, fabrication or derivation of the test item, and this information is available to the appropriate regulatory agencies should it be requested.

8.4. Analysis of Test Item

The stability of the bulk test item will not be determined during the course of this study. Information to support the stability of each lot of the bulk test item will be provided by the Sponsor.

8.5. Reserve Samples

For each batch (lot) of test item, if practically possible a reserve sample will be collected and maintained under the appropriate storage conditions by the Test Facility and destroyed after the expiration date.

8.6. Test Item Inventory and Disposition

Records of the receipt, distribution, storage, and disposition of test item will be maintained.

9. SAFETY

The following safety instructions apply to this study:

Standard safety precautions specified in Charles River Den Bosch procedures. Specific safety precautions are provided in the Charles River Den Bosch internal EH&S test item risk assessment.

10. DOSE FORMULATION AND ANALYSIS

10.1. Preparation of Test Item

No correction for the purity/composition of the test item will be performed.

Solubility of the test item in an appropriate solvent will be assessed before performing the DPRA assay. An appropriate solvent will dissolve the test item completely, i.e. by visual inspection the solution must not be cloudy nor have noticeable precipitate. Acetonitrile (ACN) is the preferred solvent for the test item. Alternatively, the following solvents may be used: Milli-Q water (MQ), ACN:MQ (1:1, v/v), isopropanol, acetone:ACN (1:1, v/v) or dimethylsulfoxide (DMSO):ACN (1:9, v/v). Other solvents can be used as long as they do not impact the stability of the peptides as monitored with reference controls C.

The test item will be pre-weighed into amber glass vials and dissolved prior testing in an appropriate volume of suitable solvent to prepare a 100 mM solution. The test item, positive control and peptide samples will be prepared less than 4 hours before starting the incubation of the cysteine (cys) or lysine (lys) reactivity assay, respectively. Visual inspection of the forming of a clear solution is considered sufficient to ascertain that the test item is dissolved.

If the test item is not soluble at a 100 mM concentration, it may still be tested at a lower soluble concentration. In such a case, a positive result can still be used to support the identification of the test item as a skin sensitizer but no firm conclusion on the lack of reactivity should be drawn from a negative result.

Any residual volumes will be discarded unless otherwise requested by the Study Director.

10.2. Sample Analysis

Analysis of test item in vehicle for concentration, stability, homogeneity will not be performed, however, to limit the impact, the test item preparation will be performed with approved procedures and documented in detail. Formulations will be visually inspected for homogeneity prior to use and all formulations will be used within 4 hours after adding vehicle to the test item. This GLP exception was therefore considered as being minor with no impact on the outcomes and the integrity and the achievement of the objective of the study.

11. TEST SYSTEM

Test system	Synthetic peptides containing cysteine (SPCC) (Ac-RFAA**C**AA-COOH) or synthetic peptides containing lysine (SPCL) (Ac-RFAA**K**AA-COOH). The molecular weight is 750.9 g/mol for SPCC, and 775.9 g/mol for SPCL.
Rationale	Recommended test system in the international OECD guideline for DPRA studies.
Source	JPT Peptide Technologies GmbH, Berlin, Germany.
Batch	Batch numbers used will be documented in the raw data and presented in the report.
Storage	The peptides will be stored in the freezer (≤-15°C) for a maximum of 6 months.

12. EXPERIMENTAL DESIGN

12.1. Preparation of Solutions for Cysteine Reactivity Assay

12.1.1. Synthetic Peptide Containing Cysteine (SPCC) Stock Solution

A stock solution of 0.667 mM SPCC (0.501 mg SPCC/mL) will be prepared by dissolving 10 mg of SPCC in 19.96 mL phosphate buffer pH 7.5 in a 20 mL glass flask. The mixture will be stirred followed by sonication. The used weight and volume may be adjusted, provided that the weight/volume ratios remain unchanged. The exact weight of the peptide and the exact volume of phosphate buffer added will be described in the report.

12.1.2. SPCC Reference Control Solutions

Three 0.5 mM SPCC reference control (RC) solutions (RCcysA, RCcysB and RCcysC) will be prepared in amber vials. RCcysA, RCcysB and RCcysC will be prepared by mixing 750 µL of the 0.667 mM SPCC stock solution with 250 µL ACN. If necessary, additional RCcysC samples will be included for each additional solvent used besides ACN. This in order to evaluate the effect of the solvent on the Percent Peptide Depletion.

12.1.3. SPCC Calibration Curve

A SPCC calibration curve will be prepared as described in the table below:

Preparation of SPCC Calibration Curve

SPCC calibration solutions	SPCC concentration (mM)*	Preparation
STDcys1	0.534	1600 µL stock solution of 0.667 mM SPCC + 400 µL ACN
STDcys2	0.267	1 mL STDcys1 + 1 mL STDcys7
STDcys3	0.134	1 mL STDcys2 + 1 mL STDcys7
STDcys4	0.067	1 mL STDcys3 + 1 mL STDcys7
STDcys5	0.033	1 mL STDcys4 + 1 mL STDcys7
STDcys6	0.017	1 mL STDcys5 + 1 mL STDcys7
STDcys7	0	8 mL phosphate buffer (pH 7.5) + 2 mL ACN

* Final concentrations may slightly differ, exact values will be included in the final report.

12.1.4. Co-elution Control, Test Item and Positive Control Samples

The co-elution control (CC) samples, test item samples and the cinnamic aldehyde positive control samples (PC) will be prepared as described in the table below:

Preparation of Co-elution Control, Test Item and Positive Control Samples

Sample	Replicates	Sample code	Preparation
Co-elution control (CC)	1	CCcys-209150/A	750 µL Phosphate buffer pH 7.5 200 µL ACN 50 µL 209150/A test solution (100 mM)
Cinnamic aldehyde (PC)	3	PCcys-1 to PCcys-3	750 µL Stock solution of 0.667 mM SPCC 200 µL ACN 50 µL Cinnamic aldehyde solution (100 mM in ACN)
Test item 209150/A	3	209150/A-cys-1 to 209150/A-cys-3	750 µL Stock solution of 0.667 mM SPCC 200 µL ACN 50 µL 209150/A test solution (100 mM)

12.2. Preparation of Solutions for Lysine Reactivity Assay

12.2.1. Synthetic Peptide Containing Lysine (SPCL) Stock Solution

A stock solution of 0.667 mM SPCL (0.518 mg SPCL/mL) will be prepared by dissolving 10 mg of SPCL in 19.31 mL of ammonium acetate buffer pH 10.2 in a 20 mL glass flask. The mixture will be stirred, followed by sonication (if necessary). The used weight and volume may be adjusted, provided that the weight/volume ratios remain unchanged. The exact weight of the peptide and the exact volume of ammonium acetate buffer added will be described in the report.

12.2.2. SPCL Reference Control Solutions

Three 0.5 mM SPCL reference control (RC) solutions (RClysA, RClysB and RClysC) will be prepared in amber vials. RClysA, RClysB and RClysC will be prepared by mixing 750 µL of the 0.667 mM SPCL stock solution with 250 µL ACN. If necessary, additional RClysC samples will be included for each additional solvent used besides ACN. This in order to evaluate the effect of the solvent on the Percent Peptide Depletion.

12.2.3. SPCL Calibration Curve

A SPCL peptide calibration curve will be prepared as described in the table below:

Preparation of SPCL Calibration Curve

SPCL calibration solutions	SPCL concentration (mM)*	Preparation
STDlys1	0.534	1600 µL stock solution of 0.667 mM SPCL + 400 µL ACN
STDlys2	0.267	1 mL STDlys1 + 1 mL STDlys7
STDlys3	0.134	1 mL STDlys2 + 1 mL STDlys7
STDlys4	0.067	1 mL STDlys3 + 1 mL STDlys7
STDlys5	0.033	1 mL STDlys4 + 1 mL STDlys7
STDlys6	0.017	1 mL STDlys5 + 1 mL STDlys7
STDlys7	0	8 mL ammonium acetate buffer (pH 10.2) + 2 mL ACN

* Final concentrations may slightly differ, exact values will be included in the final report.

12.2.4. Co-elution Control, Test Item and Positive Control Samples

The co-elution control (CC) samples, test item samples and the cinnamic aldehyde positive control samples (PC) will be prepared as described in the table below:

Preparation of Co-elution Control, Test Item and Positive Control Samples

Sample	Replicates	Sample code	Preparation
Co-elution control (CC)	1	CClys-209150/A	750 µL Ammonium acetate buffer pH 10.2 250 µL 209150/A test solution (100 mM)
Cinnamic aldehyde (PC)	3	PClys-1 to PClys-3	750 µL Stock solution of 0.667 mM SPCL 250 µL Cinnamic aldehyde solution (100 mM in ACN)
Test item 209150/A	3	209150/A-lys-1 to 209150/A-lys-3	750 µL Stock solution of 0.667 mM SPCL 250 µL 209150/A test solution (100 mM)

12.3. Sample Incubations

After preparation, the samples (reference controls, calibration solutions, co-elution control, positive controls and test item samples) will be placed in the autosampler in the dark and incubated at 25±2.5°C. The incubation time between placement of the samples in the autosampler and analysis of the first RCcysB- or RClysB-sample will be 24±2 hours. The time between the first RCcysB- or RClysB-injection and the last injection of a cysteine or lysine sequence, respectively, will not exceed 30 hours.

Prior to HPLC-PDA analysis the samples will be visually inspected. If precipitation or phase separation is observed, samples may be centrifuged (100-400 *g*). The supernatant will be transferred to a new vial. This will be recorded in the raw data and described in the report.

If a precipitation or phase separation is observed after the incubation period, peptide depletion may be underestimated and a conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result.

12.4. HPLC-PDA Analysis

SPCC and SPCL peak areas in the samples will be measured by HPLC-PDA. Sample analysis will be performed using the following system:

- Surveyor MS HPLC pump (Thermo Scientific, Breda, The Netherlands)
- MPS 3C autosampler (DaVinci, Rotterdam, The Netherlands) or HTC PAL autosampler (DaVinci, Rotterdam, The Netherlands)
- LC Column oven 300 (Thermo Scientific) or Column Oven #151006 (Grace, Worms, Germany)
- Surveyor PDA detector (Thermo Scientific)

All samples will be analyzed according to the HPLC-PDA method presented in ATTACHMENT C. The HPLC sequences of the cysteine and lysine reactivity assay for the test item are presented in ATTACHMENT D.

13. ACCEPTABILITY CRITERIA

The following criteria should be met for a run to be considered valid:

a) The standard calibration curve should have an $r^2>0.99$.

b) The mean Percent Peptide Depletion value of the three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for SPCC and between 40.2% and 69.0% for SPCL.

c) The maximum standard deviation (SD) for the positive control replicates should be <14.9% for the Percent Cysteine Peptide Depletion and <11.6% for the Percent Lysine Peptide Depletion.

d) The mean peptide concentration of Reference Controls A should be 0.50 ± 0.05 mM.

e) The Coefficient of Variation (CV) of peptide areas for the nine Reference Controls B and C in ACN should be <15.0%.

The following criteria should be met for a test item's results to be considered valid:

a) The maximum standard deviation for the test item replicates should be <14.9% for the Percent Cysteine Depletion and <11.6% for the Percent Lysine Depletion.

b) The mean peptide concentration of the three Reference Controls C in the appropriate solvent should be 0.50±0.05 mM.

If (one of) the acceptability criteria are not met and the Study Director decides that this has a critical effect on the study, the test will be rejected and repeated.

14. ANALYSIS

14.1. Data Evaluation

The concentration of SPCC or SPCL will be photometrically determined at 220 nm in each sample by measuring the peak area of the appropriate peaks by peak integration and by calculating the concentration of peptide using the linear calibration curve derived from the standards.

The Percent Peptide Depletion will be determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant reference controls C according to the following formula:

$$\text{Percent Peptide Depletion} = \left[1 - \left(\frac{\text{Peptide Peak Area in Replicate Injection (at 220 nm)}}{\text{Mean Peptide Peak Area in Reference Controls (at 220 nm)}}\right)\right] \times 100$$

In addition, the absorbance at 258 nm will be determined in each sample by measuring the peak area of the appropriate peaks by peak integration. The ratio of the 220 nm peak area and the 258 nm peak will be used as an indicator of co-elution. For each sample, a ratio in the range of 90%<mean area ratio of control samples<110% would give a good indication that co-elution has not occurred. However, calculation of peak purity (area ratio 220 nm/258 nm) might not always be possible, particularly if the test item is highly reactive with the peptide.

14.2. Data Interpretation

The mean Percent Cysteine Depletion and Percent Lysine Depletion will be calculated for the test item. Negative depletion is considered as "0" when calculating the mean. By using the Cysteine 1:10 / Lysine 1:50 prediction model (see table below), the threshold of 6.38% average peptide depletion will be used to support the discrimination between a skin sensitizer and a non-sensitizer.

Cysteine 1:10 / Lysine 1:50 Prediction Model

Mean of cysteine and lysine % depletion	Reactivity class	DPRA prediction
0% ≤ Mean % depletion ≤ 6.38%	No or minimal reactivity	Negative
6.38% < Mean % depletion ≤ 22.62%	Low reactivity	Positive
22.62% < Mean % depletion ≤ 42.47%	Moderate reactivity	
42.47% < Mean % depletion ≤ 100%	High reactivity	

There might be cases where the test item absorbs significantly at 220 nm and has the same retention time as the peptide (co-elution). If co-elution of a test item occurs with both the cysteine and the lysine peptide then the analysis will be reported as "inconclusive". In the case where co-elution occurs only with the lysine peptide, the Cysteine 1:10 prediction model will be used (see table below).

Cysteine 1:10 Prediction Model

Cysteine (Cys) % depletion	Reactivity class	DPRA prediction
0% ≤ Cys % depletion ≤ 13.89%	No or minimal reactivity	Negative
13.89% < Cys % depletion ≤ 23.09%	Low reactivity	Positive
23.09% < Cys % depletion ≤ 98.24%	Moderate reactivity	
98.24% < Cys % depletion ≤ 100%	High reactivity	

In case the overlap in retention time between the test item and either of the peptides is incomplete, the Percent Peptide Depletion will be estimated and used in the Cysteine 1:10 / Lysine 1:50 prediction model. However, assignment to a reactivity class will not be made.

15. COMPUTERIZED SYSTEMS

The following critical computerized systems may be used in the study. The actual critical computerized systems used will be specified in the Final Report.

Data for parameters not required by study plan, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by study plan and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

Critical Computerized Systems

System Name	Description of Data Collected and/or Analyzed
REES Centron	Temperature, relative humidity and/or atmospheric pressure monitoring
Xcalibur	System control, data acquisition and integration

16. AMENDMENTS AND DEVIATIONS

Changes to the approved study plan shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary study plan changes in advance with the Sponsor.

All study plan and SOP deviations will be documented in the study records. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

17. RETENTION OF RECORDS

All study-specific raw data, electronic data, documentation, study plan and final reports will be archived by no later than the date of final report issue. All materials generated by Charles River from this study will be transferred to a Charles River archive. At least five years after issue of the final report, the Sponsor will be contacted.

Records to be maintained will include, but will not be limited to, documentation and data for the following:

- Study plan, study plan amendments, and deviations
- Study schedule
- Study-related correspondence
- Test system receipt
- Test item receipt, identification and preparation
- Measurements and observations

18. REPORTING

A comprehensive Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft Report. The Final Report will be provided in Adobe Acrobat PDF format (hyperlinked and searchable) along with a Microsoft Word version of the text. The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Test Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation. An original copy of the report with the Test Facility's handwritten signatures will be retained.

Reports should be finalized within 6 months of issue of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Test Facility unless other arrangements are made by the Sponsor.

TEST FACILITY APPROVAL

The signature below acknowledges Test Facility Management's responsibility to the study as defined by the relevant GLP regulations.

M.A.M. Wenker, PhD, ERT
Section Head Drug Metabolism and Pharmacokinetics

Date: 29 March 2018

The signature below indicates that the Study Director approves the study plan.

Date: 29 March 2018

J. Reinen, PhD
Study Director

ATTACHMENT A

Distribution List

Electronic copies will be supplied unless otherwise specified below.

Version	Recipient
Original	Study Director
1 Copy	Sponsor Representative / Study Monitor
1 Copy	QAU / Management

ATTACHMENT B

Certificate of Analysis

2

ATTACHMENT C

Method for HPLC-PDA Analysis

HPLC-PDA Method for Determination of SPCC and SPCL

Mobile phase	A: 0.1% (v/v) TFA in Milli-Q water B: 0.085% (v/v) TFA in ACN	
Gradient	Cysteine	Lysine
	0 min 10% B 10 min 25% B 11 min 90% B 13 min 90% B 13.5 min 10% B 20 min 10% B	0 min 10% B 10 min 20% B 11 min 90% B 13 min 90% B 13.5 min 10% B 20 min 10% B
Flow	0.35 mL/min	
Injection volume	3–10 µL	
Sample tray temperature	Set at 25°C	
Column	Zorbax SB-C_{18}, 100 mm x 2.1 mm, df = 3.5 µm (Agilent Technologies, Santa Clara, CA, USA)	
Guard column	SecurityGuard™ cartridge for C_{18}, 4 x 2.0 mm (Phenomenex, Torrance, CA, USA)	
Column temperature	Set at 30°C	
Detection	Photodiode array detection, monitoring at 220 and 258 nm	

ATTACHMENT D

HPLC-PDA Analysis Sequences

Analysis Sequences for Cysteine and Lysine Reactivity Assays

Sample description	Cysteine reactivity assay	Lysine reactivity assay
Calibration standards (STD) and reference controls (RC)	STDcys7 STDcys6 STDcys5 STDcys4 STDcys3 STDcys2 STDcys1 Dilution buffer RCcysA-1 RCcysA-2 RCcysA-3	STDlys7 STDlys6 STDlys5 STDlys4 STDlys3 STDlys2 STDlys1 Dilution buffer RClysA-1 RClysA-2 RClysA-3
Co-elution controls (CC)	CCcys-209150/A	CClys-209150/A
Reference controls (RC)	RCcysB-1 RCcysB-2 RCcysB-3	RClysB-1 RClysB-2 RClysB-3
First set of replicates	RCcysC-1[1)] PCcys-1 209150/A-cys-1	RClysC-1[1)] PClys-1 209150/A-lys-1
Second set of replicates	RCcysC-2[1)] PCcys-2 209150/A-cys-2	RClysC-2[1)] PClys-2 209150/A-lys-2
Third set of replicates	RCcysC-3[1)] PCcys-3 209150/A-cys-3	RClysC-3[1)] PClys-3 209150/A-lys-3
Reference controls	RCcysB-4 RCcysB-5 RCcysB-6	RClysB-4 RClysB-5 RClysB-6

STD = standard; RC = reference control; CC = co-elution control; PC = positive control.
[1)] For each solvent used, a RCcys and RClys sample will be included in the analysis sequence.

DEVIATIONS

All deviations that occurred during the study have been authorized/acknowledged by the Study Director, assessed for impact, and documented in the study records. All study plan deviations and those SOP deviations that could have impacted the quality or integrity of the study are listed below.

None of the deviations were considered to have impacted the overall integrity of the study or the interpretation of the study results and conclusions.

Test Item Concentration used for Solubility Assessment

-

  Evaluation: The solubility was performed at a higher concentration (110 mM instead of the intended 100 mM) and as a result the outcome of the solubility test can still be used to select the appropriate solvent for the DPRA study. Therefore, this deviation has no impact on the integrity of the study.

Appendix 6
Test and Reference Item Characterization

Certificate of Analysis of

Certificate of analysis of cinnamic aldehyde (RS473/A)

SIGMA-ALDRICH®

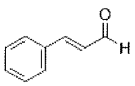
sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Cinnamaldehyde - natural, ≥95%, FG

Product Number:	**W228613**
Batch Number:	**MKCB9907**
Brand:	ALDRICH
CAS Number:	104-55-2
MDL Number:	MFCD00007000
Formula:	C9H8O
Formula Weight:	132.16 g/mol
Quality Release Date:	08 DEC 2016
Expiration Date:	NOV 2021



Test	Specification	Result
Appearance (Color)	Colorless to Yellow	Colorless
Appearance (Form)	Liquid	Liquid
Refractive index at 20 ° C	1.614 - 1.623	1.622
Infrared Spectrum	Conforms to Structure	Conforms
Purity (GC)	≥ 95.0 %	99.1 %
Specific Gravity	1.046 - 1.052	1.048
Acid Value	≤ 5.0 ml	1.5 ml
Arsenic (As)	≤ 3 ppm	< 1 ppm
Cadmium (Cd)	≤ 1 ppm	< 1 ppm
Mercury (Hg)	≤ 1 ppm	< 1 ppm
Lead (Pb)	≤ 10 ppm	< 1 ppm
Expiration Date Period 5 Years	----------	----------

Michael Grady, Manager
Quality Control
Milwaukee, WI US

Appendix 7
Analytical Data Sheets of SPCC and SPCL

Analytical data sheet of SPCC

Analytical Data Sheet

Innovative Peptide Solutions

JE.#	35144_2
PO.#	6600504743
Customer	Charles River Mr. Rody van Haren
Product	Cysteine peptide
Sequence	Ac-RFAACAA-OH
Amount	210.2 mg
Batch no.	111016HS_MHeW0218
Molecular weight (net)	750.88 g/mol (average)
Specification	>98% (HPLC - 220nm - C18 - linear gradient)

Tests	**Results**		
Appearance	lyophilized material		
MS [m/z]	751.4	[M+H]+	ESI
	1501.7	[2M+H]+	ESI
	-	-	
Purity found [%]	98.08 (see raw data enclosed)		
AAA calcd.	n.d.		
AAA found	n.d. (Non-normalized, see raw data enclosed. Amino acids labeled with an asterisk could not be properly determined due to incomplete hydrolysis or decomposition. Such amino acids were excluded from determination of the peptide content.)		
Peptide content [%]	n.d.		
Remark	This product is supplied as a trifluoroacetate salt.		
Storage conditions:	minus 20 °C Use recommended within 6 months from date of quality control.		
Date	February 23, 2018		
Approved by	HENKEL (Analytical Data see next pages)		

Geschäftsführer / Managing Directors
Dr. Holger Wenschuh
Sean Marett

Sitz / Registered Office Berlin
Gerichtsstand / Jurisdiction Berlin
Amtsgericht Charlottenburg
HRB 92692 B
Ust.-IdNr. / VAT-ID DE814044483

Bankverbindung / Bank Account
Deutsche Bank Berlin
Konto-Nr. / Account-No. 915 165 500
IBAN DE84 1007 0000 0915 1655 00
BIC / SWIFT DEUTDEBBXXX

JPT Peptide Technologies GmbH
Volmerstrasse 5
12489 Berlin, Germany
T +49-30-6392-5500
peptide@jpt.com | www.jpt.com

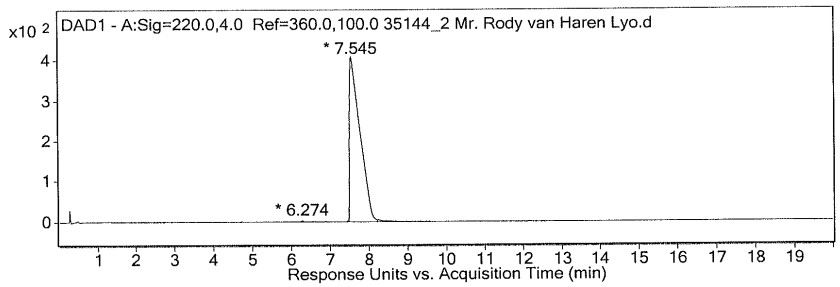
Analytical data sheet of SPCC *(continued)*

Innovative Peptide Solutions

Data Filename	35144_2 Mr. Rody van Haren Lyo.d	**Sample Name**	35144_2
Sample Type	Sample	**Position**	P1-D1
Instrument Name	Instrument 1	**User Name**	Dirk Wildemann
Acq Method	hydrophile Peptide.m	**Acquired Time**	2/14/2018 2:55:34 PM
DA Method	peptid auswertung neu.m	**Comment**	35144_2 Mr. Rody van Haren Lyo

35144_2
Mr. Rody van Haren
Cysteine peptide
Ac-RFAACAA-OH
batch No. 111016HS_MHeW0218
210.20 mg

User Chromatograms

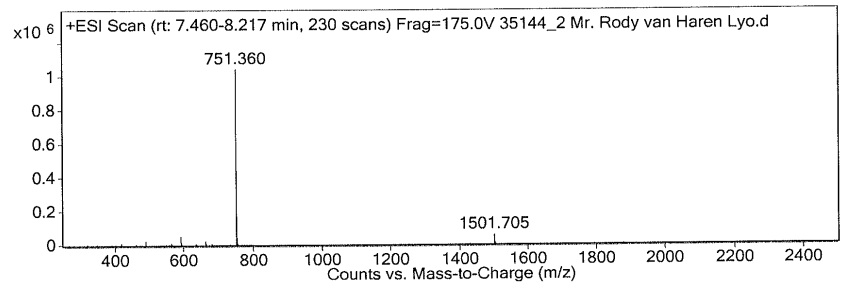


Integration Peak List

Peak	Start	RT	End	Height	Area	AreaSumPercent
1	6.169	6.274	6.351	3.27	11.15	0.14
2	6.351	6.375	6.836	0.63	8.96	0.11
3	6.836	6.877	6.951	1.2	4.63	0.06
4	6.951	7.488	7.488	29.78	20.41	0.26
5	7.488	7.545	8.218	408.36	7671.97	98.08
6	8.218	8.25	10.161	5.38	105.04	1.34

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
175	0	ESI



--- End Of Report ---

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Analytical data sheet of SPCL

Analytical Data Sheet

Innovative Peptide Solutions

JE.#	35144_1
PO.#	6600504743
Customer	Charles River Mr. Rody van Haren
Product	Lysine peptide
Sequence	Ac-RFAAKAA-OH
Amount	210.4 mg
Batch no.	120514HSDWW0218
Molecular weight (net)	775.91 g/mol (average)
Specification	>98% (HPLC - 220nm - C18 - linear gradient)

Tests	**Results**		
Appearance	lyophilized material		
MS [m/z]	776.4	[M+H]+	ESI
	388.7	[M+2H]2+	ESI
	-	-	
Purity found [%]	99.28 (see raw data enclosed)		
AAA calcd.	n.d.		
AAA found	n.d. (Non-normalized, see raw data enclosed. Amino acids labeled with an asterisk could not be properly determined due to incomplete hydrolysis or decomposition. Such amino acids were excluded from determination of the peptide content.)		
Peptide content [%]	n.d.		
Remark	This product is supplied as a trifluoroacetate salt.		
Storage conditions:	minus 20 °C Use recommended within 6 months from date of quality control.		
Date	February 23, 2018		
Approved by	HENKEL (Analytical Data see next pages)		

Geschäftsführer / Managing Directors
Dr. Holger Wenschuh
Sean Marett

Sitz / Registered Office Berlin
Gerichtsstand / Jurisdiction Berlin
Amtsgericht Charlottenburg
HRB 92692 B
Ust.-IdNr. / VAT-ID: DE814044483

Bankverbindung / Bank Account
Deutsche Bank Berlin
Konto-Nr. / Account-No. 915 165 500
IBAN DE84 1007 0000 0915 1655 00
BIC / SWIFT DEUTDEBBXXX

JPT Peptide Technologies GmbH
Volmerstrasse 5
12489 Berlin, Germany
T +49-30-6392-5500
peptide@jpt.com | www.jpt.com

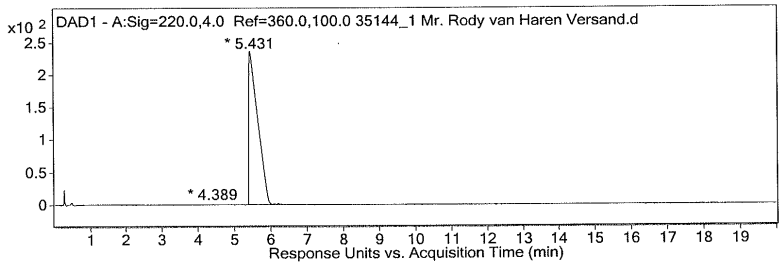
Analytical data sheet of SPCL *(continued)*

Innovative Peptide Solutions

Data Filename	35144_1 Mr. Rody van Haren Versand.d	**Sample Name**	35144_1
Sample Type	Sample	**Position**	P1-B6
Instrument Name	Instrument 1	**User Name**	Dirk Wildemann
Acq Method	hydrophile Peptide.m	**Acquired Time**	2/13/2018 1:24:09 PM
DA Method	peptid auswertung neu.m	**Comment**	35144_1 Mr. Rody van Haren Versand

35144_1
ID: 662534
Mr. Rody van Haren
Lysine peptide
Ac-RFAAKAA-OH
batch No. 120514HSD/MM0219
210.40 mg

User Chromatograms

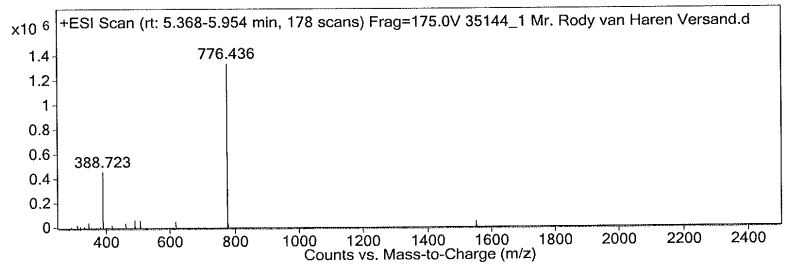


Integration Peak List

Peak	Start	RT	End	Height	Area	AreaSumPercent
1	4.162	4.389	4.533	0.43	3.17	0.08
2	4.998	5.38	5.38	19.93	6.83	0.17
3	5.38	5.431	6.047	236.92	4059.6	99.28
4	6.047	6.201	6.697	2.04	19.32	0.47

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
175	0	ESI



--- End Of Report ---

Printed at: 1:49 PM on: 2/13/2018

charles river

SUMMARY REPORT
(EARLY TERMINATION OF STUDY)

Test Facility Study No. 20146047

Evaluation of the in vitro Skin Sensitization Potential of with the KeratinoSens™ Assay

TEST FACILITY:
Charles River Laboratories Den Bosch B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

The objective of this study was to evaluate the ability of to activate the antioxidant/electrophile responsive element (ARE)-dependent pathway in the KeratinoSens™ assay.

clear colourless liquid.

is not soluble in the standard solvents dimethyl sulfoxide (Uvasol, Merck, Darmstadt, Germany), Milli-Q water (Millipore Corp., Bedford, MA., USA) and ethanol (Lichrosolv, Merck, Darmstadt, Germany). In addition, a homogenous suspension could not be obtained in these solvents.

Therefore it is concluded that the test item is not suitable to perform in this test. So, no conclusion can be made based on the obtained data.

Charles River Den Bosch

Signature: .....

Name: J.A.J. Woutersen, MSc

Title: Study Director

Date: 29 Jun 2018

This summary report was inspected by the Test Facility Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s).

The dates of Quality Assurance inspections are given below.

Test Facility study number 20146047

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date to TFM and SD*
Study	Final study plan	20/Apr/2018	20/Apr/2018	20/Apr/2018
	Study Plan Amendment 01	6/Jun/2018	6/Jun/2018	6/Jun/2018
	Summary Report	7/Jun/2018	7/Jun/2018	11/Jun/2018
	Final Summary Report	21/Jun/2018	21/Jun/2018	22/Jun/2018
Process	**Genetic and In Vitro Toxicology**	19/Feb/2018	2/Mar/2018	2/Mar/2018
	Exposure			
	Observations/Measurements			
	Specimen Handling			
	Test Item Handling			
	Test Item Receipt	13/Mar/2018	21/Mar/2018	21/Mar/2018
	Test Item Handling			

Date: 29 Jun 2018

Samantha Leo-Vos
Quality Assurance Auditor

TRADE SECRET

Study Title

CAS # READY BIODEGRADABILITY-CO_2 IN SEALED VESSELS (HEADSPACE TEST)

Test Guidelines

Organisation for Economic Cooperation and Development
OECD Guideline 310

Authors

Edward C. Schaefer, B.S.
Ning Wang, Ph.D.
Asmaa Muneer, B.S.

Date Study Completed

July 31, 2018

Test Facility

EAG, Inc.
8598 Commerce Drive
Easton, Maryland 21601 USA
1 (410) 822-8600

Project Identification Numbers

EAG Laboratories Study No.: 783E-106

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The study described in this final report was conducted in compliance with U.S. EPA Good Laboratory Practice Standards (40 CFR part 792) that are compatible with OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98) 17).

The stability of the reference substance under storage condition at the test site was not determined in accordance with Good Laboratory Practice Standards.

This study was also conducted as per the mutually agreed study protocol and test facility's Standard Operating Procedures.

Study Director

Ning Wang, Ph.D.
Principal Scientist
EAG Laboratories-Easton

31 July 2018
Date

QUALITY ASSURANCE STATEMENT

CAS READY BIODEGRADABILITY-CO_2 IN SEALED VESSELS (HEADSPACE TEST)

The raw data and final report for this study were inspected by the EAG Laboratories Quality Assurance Unit (QAU) to assure compliance with the study protocol, standard operating procedures and the pertinent EPA Good Laboratory Practice Regulations which are compatible with the OECD Principles of Good Laboratory Practice. Dates of study inspections and the dates reported to the Study Director and Management are listed below.

EAG Laboratories QAU Study-Specific Audits

Phase Inspected	Date Inspected/Audited	Date Reported to Study Director	Date Reported to Management
Protocol	February 14, 2018	February 14, 2018	June 28, 2018
Test Substance Administration	April 25, 2018	April 25, 2018	April 26, 2018
NaOH addition	May 18, 2018	May 18, 2018	May 21, 2018
Data and Draft Report	July 6-9, 2018	July 9, 2018	July 20, 2018
Final Report	July 31, 2018	July 31, 2018	July 31, 2018

Angela Hengst
Senior Quality Assurance Representative
EAG Laboratories-Easton

July 31, 2018
Date

CERTIFICATION

CAS READY BIODEGRADABILITY-CO_2 IN SEALED VESSELS (HEADSPACE TEST)

The Study Director hereby declares that the work was performed under his supervision and in accordance with the described procedures. It is assured that the reported results faithfully represent the raw data obtained during the experimental work. No circumstances have been left unreported, which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study.

The Study Director accepts overall responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results.

Report by:

Ning Wang, Ph.D.
Principal Scientist, Environmental Fate
EAG Laboratories-Easton

31 July 2018
Date

Approved by:

Edward C. Schaefer, B.S.
Director of Environmental Fate
EAG Laboratories-Easton

31 July 2018
Date

TABLE OF CONTENTS

TABLES

FIGURE

APPENDICES

BASIC STUDY INFORMATION

Study Title:	CAS # Ready Biodegradability- CO_2 in Sealed Vessels (Headspace Test)
EAG Laboratories Study No.:	783E-106
Type of Report:	Final
Type of Study:	Ready Biodegradability
Test Item:	CAS #
Authors:	Edward C. Schaefer, B.S., Director of Environmental Fate Ning Wang, Ph.D., Principal Scientist Asmaa Muneer, B.S., Associate Scientist II
Study Initiation Date:	April 02, 2018
Experimental Start Date (OECD):	April 02, 2018
Experimental Start Date (EPA):	April 25, 2018
Experimental Completion Date:	May 24, 2018
Study Completion Date:	July 31, 2018
Study Director:	Ning Wang, Ph.D., Principal Scientist, Environmental Fate EAG Laboratories
Principal Analyst	Asmaa Muneer, B.S., Associate Scientist II, Environmental Fate EAG Laboratories

STUDY PERSONNEL

The following personnel participated in the conduct of the study:

Edward C. Schaefer, B.S., Director of Environmental Fate
Ning Wang, Ph.D., Principal Scientist, Environmental Fate
Asmaa Muneer, B.S., Associate Scientist II, Environmental Fate

CAS # READY BIODEGRADABILITY-CO_2 IN SEALED VESSELS (HEADSPACE TEST)

1.0 EXECUTIVE SUMMARY

The ready biodegradability of CAS # was determined by the Headspace Test (according to OECD Guideline 310 (1)). Tests of ready biodegradability are stringent tests that provide limited opportunity for acclimation and biodegradation to occur. In the Headspace Test, inoculated test medium was dosed with a known amount of test substance as the nominal sole source of organic carbon and sealed. CO_2 evolution from the ultimate aerobic biodegradation of the test substance is determined by measuring the inorganic carbon (IC) produced in the test bottles over that produced in the blank control bottles. The amount of IC produced by the test substance (corrected for that evolved by the controls and the amount added by basification, where applicable) is expressed as a percentage of the theoretical amount of IC (ThIC) that could have been produced if complete biodegradation of the test substance occurred. The test contained a control group, a reference group and a treatment group. Each group contained twenty-nine replicate test chambers. The controls were used to measure the background IC production of the inoculum medium and were not dosed with a carbon source. The reference chambers were dosed with 1-octanol, a substance known to be biodegradable, at a nominal concentration of 20 mg C/L. The treatment group test chambers were used to evaluate CAS # at a nominal concentration of approximately 20 mg C/L. The results at the end of the 28-day study indicated that the activated sludge inoculum was active, degrading the reference substance 93.5%. The average cumulative percent biodegradation for CAS # was 0.3%.

Test Substance	Average Cumulative Percent Biodegradation	Readily Biodegradable[1]
CAS #	0.3	No

[1] Meets or exceeds the OECD criteria for ready biodegradability (60% of ThIC within a 10-day window of reaching 10% ThIC).

2.0 INTRODUCTION

Tests of ready biodegradability, by definition, provide limited opportunity for acclimation and biodegradation to occur. A positive result in a test of ready biodegradability is an indication that the test substance will undergo rapid and ultimate biodegradation in the environment. A negative result in a test of ready biodegradability does not necessarily mean that the test substance will not be biodegraded under relevant environmental conditions but that additional testing may be needed.

This study was conducted by EAG Laboratories for at the EAG Laboratories biodegradation facility in Easton, Maryland. Original raw data generated by EAG Laboratories and a copy of the final report are filed under project number 783E-106 in the archives located on the EAG Laboratories site.

2.1 Objective of Study

The objective of the study was to assess the biodegradability of the test substance in an aqueous medium by measuring inorganic carbon (IC) production.

2.3 Guidelines

The study design was based on the procedures specified in the OECD Guideline 310 (1) and ECHA Guidance (R7.9.4 and R7.9.5 –Evaluation of available information on degradation/biodegradation and Conclusions for degradation/biodegradation in Chapter R.7B –Endpoint Specific Guidance) (2).

3.0 MATERIALS AND METHODS

This study was conducted according to the procedures outlined in the protocol, "CAS #41890-92-0: Ready Biodegradability-CO2 in Sealed Vessels (Headspace Test)". The protocol was based on the procedures specified in the OECD Guideline 310 (1) and ECHA Guidance (R7.9.4 and R7.9.5 –Evaluation of available information on degradation/biodegradation and Conclusions for degradation/biodegradation in Chapter R.7B –Endpoint Specific Guidance) (2).

3.1 Test and Reference Items

3.1.1 *Test Item*

The test substance was received from on April 06, 2018 and was assigned EAG Laboratories identification number 14633. The following is a description of the test substance used in this study and a Certificate of Analysis can be found in Appendix I:

Identity:

CAS Number:

Lot Number: 12639

Physical Description:	Liquid
Purity:	99.09%
Expiration Date:	04/05/2020
Storage Conditions:	Ambient

The test substance was administered to the treatment group test chambers by direct weight addition. Direct weight addition of a test substance that is relatively insoluble in water or of unknown solubility is the most appropriate route of administration. Dosing amounts were based on the calculated carbon content of the test substance.

3.1.2 *Reference Item*

The reference substance was received from Sigma Aldrich on April 02, 2018 and was assigned EAG Laboratories Identification number 14605. The following is a description of the reference substance used in this study:

Name:	1-Octanol
Batch Number:	BCBT3395
Physical Description:	Liquid
Chemical Abstract Number:	111-87-5
Expiration Date:	Nov 2019
Purity:	99.7%
Storage Conditions:	Ambient
Molecular Formula:	$CH_3(CH_2)_7OH$
Molecular Weight:	130.23 g/mol
Carbon Content:	73.78% (Calculated based on molecular formula)

The reference substance was administered to the treatment group test chambers by direct weight addition. Direct weight addition of a test substance that is relatively insoluble in water or of unknown solubility is the most appropriate route of administration. Dosing amounts were based on the calculated carbon content of the reference substance.

3.2 ***Test Medium***

The test medium was a mineral salts media and was prepared using high quality water. All chemicals and reagents used in the preparation of the test medium were reagent grade or better. The constituents of the test medium are not known to contain any contaminants that are reasonably expected to be present or known to be capable of

interfering with the study.

3.3 Test Apparatus and Conditions

The test chambers were glass serum bottles with a nominal volume of 160 mL. The chambers were sealed with butyl rubber septa and crimp caps. The test was conducted at 20 ± 3ºC. The test chambers were mixed at a rate sufficient to keep the bottle contents mixed and in suspension throughout the study. Test chambers were identified by project number, test substance ID, test concentration and replicate.

3.4 Test Inoculum

Activated sludge was collected from the Easton Wastewater Treatment Facility, Easton, Maryland on April 24, 2018. The Easton facility treats predominantly residential wastes. The sludge was sieved using a 2-mm screen and then aerated until its adjustment.

3.5 Preconditioning of Inoculum

The activated sludge was diluted in test medium to approximately 30 mg total suspended solids/L and aerated with CO_2-free air until its dilution.

3.6 Inoculated Medium

The inoculated medium was prepared by diluting the preconditioned activated sludge in test medium to approximately 4 mg total suspended solids/L. After adjustment, the sludge was aerated for approximately 30 minutes. Following aeration, the pH (7.5), dissolved organic carbon (DOC = 0.25 mg/L) and dissolved inorganic carbon (DIC = 0.0 mg/L) levels of the mixture were measured.

3.7 Preparation of Test Chambers

Aliquots of inoculated test medium were dispensed into 160 mL replicate bottles to give a headspace to liquid ratio of approximately 1:2 (107 mL in a 160 mL bottle). The blank controls contained only inoculated medium and were not dosed with a carbon source. Reference group test chambers were dosed with sufficient reference substance (2.9 mg) necessary to deliver 20 mg C/L. Treatment group test chambers were dosed with sufficient test substance (10.4 mg) necessary to deliver 20 mg C/L.

3.8 Sample Collection and Analysis

Three replicate chambers from each group were removed for IC analysis twice a week over the test period except for day 28 of the test where 5 replicate chambers were analyzed from each group.

Samples for analysis were prepared by converting CO_2 to carbonate. One milliliter of 7M NaOH was injected into the test chambers, which were subsequently shaken for at least 1 hour at test temperature. After mixing, the chambers were removed from the shaker table, allowed to settle and then samples were removed for IC analysis. At each interval, the NaOH used was analyzed for its IC concentration.

3.9 Calculations

The total mass (mg) of inorganic carbon at each sampling interval was calculated using the following equation:

TIC = (mg IC/L in vessel – mg IC/L added from 7M NaOH) × volume of liquid in vessel

The percent degradation at each sampling interval was calculated using the following equation:

$$D_t = [TIC_t - TIC_B] / TOC \times 100$$

D_t = % degradation at time t

TIC_t = mg TIC in test chamber at time t

TIC_B = mean mg TIC in blank at time t

TOC = mg TOC added initially to the test chamber

4.0 RESULTS AND DISCUSSION

4.1. Observations and Measurements

The temperature range recorded during the test was 19.50 to 23.60ºC. The mean initial TSS of the activated sludge measured 6213 prior to adjustment. The measured IC concentrations of the NaOH basification solutions used at each sampling interval are presented in Table 1. The measured concentrations of inorganic carbon in the test chambers are presented in Table 2. The concentration of inorganic carbon in test chambers adjusted for amount of carbon added during basification are presented in Table 3. The total mass of inorganic carbon measured in the test chambers are presented in Table 4. The percent degradation of the test chambers are presented in Table 5. The average percent degradation at each sampling interval is presented in graphical form in Figure 1.

The control chambers evolved an average of 0.86 mg IC/L by Day 28. The amount of IC evolved by the control chambers was < 15% of the organic carbon added initially as the test substance (20 mg/L), which is considered the acceptable limit for this study.

The viability of the inoculum and validity of the test were supported by the reference substance, 1-Octanol, degrading an average of 93.5 ± 5.4% by day 28 (Table 5). An average percent biodegradation of greater than 60% was achieved by Day 14, thereby fulfilling the criteria for a valid test by reaching the pass level by Day 28. The average cumulative percent biodegradation for CAS # was 0.3 ± 1.9% (Table 5). CAS # may not be considered readily biodegradable under aerobic conditions since the pass level of 60% ThIC was not achieved.

5.0 CONCLUSIONS

The average cumulative percent biodegradation for CAS # was 0.3%. CAS # may not be considered readily biodegradable under aerobic conditions since the pass level of 60% ThIC within a 10-day window of reaching 10% ThIC was not achieved during the test.

6.0 RETENTION OF RECORDS (ARCHIVING)

Original raw data generated by EAG Laboratories and a copy of the final report will be transferred to at study finalization.

7.0 REFERENCES

1. **Organisation for Economic Cooperation and Development**. 2006. Ready Biodegradability-CO_2 in Sealed Vessels (Headspace Test). OECD Guideline 310.

2. **ECHA Guidance** (R7.9.4 and R7.9.5 –*Evaluation of available information on degradation/biodegradation* and *Conclusions for degradation/biodegradation* in Chapter R.7B – Endpoint Specific Guidance).

TABLE 1 MEASURED CONCENTRATIONS OF NAOH SOLUTIONS

Interval (Day)	IC (mg IC/L)
3	85.02
6	83.26
9	74.15
12	71.89
16	71.3
20	128.5
23	148.3
26	114.4
28	106.1

TABLE 2 MEASURED INORGANIC CARBON CONCENTRATION OF TEST CHAMBERS (MG C/L)

Date	Day	Control Replicate #	Result	1-Octanol Replicate #	Result	Replicate #	Result
28-Apr-18	3	1	1.5	1	8.9	1	1.5
28-Apr-18	3	2	1.4	2	11.1	2	1.3
28-Apr-18	3	3	1.8	3	11.5	3	1.7
1-May-18	6	4	1.8	4	19.5	4	1.8
1-May-18	6	5	1.8	5	18.0	5	1.8
1-May-18	6	6	2.1	6	16.5	*6	--
4-May-18	9	7	1.6	7	17.2	7	1.7
4-May-18	9	8	1.6	8	19.8	8	1.7
4-May-18	9	9	1.8	9	17.7	9	1.8
7-May-18	12	10	2.0	10	20.7	10	1.5
7-May-18	12	11	1.5	11	16.4	11	1.5
7-May-18	12	12	1.9	12	21.0	12	1.9
11-May-18	16	13	1.6	13	18.3	13	1.6
11-May-18	16	14	1.6	14	20.3	14	1.5
11-May-18	16	15	1.8	15	19.4	15	1.8
15-May-18	20	16	2.1	16	20.9	16	2.1
15-May-18	20	17	2.4	17	21.5	17	1.9
15-May-18	20	18	2.4	18	21.3	18	2.3
18-May-18	23	19	1.8	19	23.1	19	1.7
18-May-18	23	20	1.8	20	22.4	20	1.8
18-May-18	23	21	1.9	21	22.1	21	2.0
21-May-18	26	22	2.1	22	18.5	22	2.2
21-May-18	26	23	2.1	23	20.6	23	2.0
21-May-18	26	24	2.3	24	18.2	24	2.3
23-May-18	28	25	1.7	25	20.3	25	1.7
23-May-18	28	26	1.6	26	21.5	26	1.7
23-May-18	28	27	1.9	27	19.8	27	1.6
23-May-18	28	28	1.6	28	19.0	28	1.7
23-May-18	28	29	2.3	29	21.8	29	2.5

"Result" values were rounded for presentation purpose. * Sample was not analyzed as the vial was broken during analysis.

TABLE 3 INORGANIC CARBON CONCENTRATIONS ADJUSTED FOR AMOUNT OF CARBON ADDED DURING BASIFICATION (MG C/L)

Date	Day	Replicate #	Control Result	1-Octanol Result	Result
28-Apr-18	3	1	0.7	8 1	0.7
28-Apr-18	3	2	0.6	10.3	0.5
28-Apr-18	3	3	1.0	10.7	0.9
1-May-18	6	4	1.0	18.7	1.0
1-May-18	6	5	1.0	17.2	1.0
1-May-18	6	6	1.3	15.7	-0.8
4-May-18	9	7	1.0	16.5	1.0
4-May-18	9	8	0.9	19.1	1.0
4-May-18	9	9	1.1	17.0	1.1
7-May-18	12	10	1.3	20.0	0.8
7-May-18	12	11	0.9	15.7	0.8
7-May-18	12	12	1.2	20.3	1.2
11-May-18	16	13	0.9	17.6	0.9
11-May-18	16	14	0.9	19.6	0.9
11-May-18	16	15	1.1	18.8	1.1
15-May-18	20	16	0.9	19.7	0.9
15-May-18	20	17	1.2	20.3	0.7
15-May-18	20	18	1.2	20.0	1.1
18-May-18	23	19	0.5	21.7	0.4
18-May-18	23	20	0.4	21.0	0.4
18-May-18	23	21	0.5	20.8	0.6
21-May-18	26	22	1.0	17.4	1.2
21-May-18	26	23	1.0	19.6	1.0
21-May-18	26	24	1.2	17.1	1.2
23-May-18	28	25	0.8	19.3	0.7
23-May-18	28	26	0.6	20.5	0.7
23-May-18	28	27	1.0	18.8	0.6
23-May-18	28	28	0.6	18.0	0.7
23-May-18	28	29	1.3	20.8	1.5

Values are adjusted for the amount of carbon added during basification. ”Result” values were rounded for presentation purpose.

TABLE 4 TOTAL MASS OF INORGANIC CARBON (MG)[1,2]

Date	Day	Control Replicate #	Result	1-Octanol Replicate #	Result	Replicate #	Result
28-Apr-18	3	1	0 1	1	0.8	1	0.1
28-Apr-18	3	2	0 1	2	1.0	2	0.1
28-Apr-18	3	3	0 1	3	1.1	3	0.1
1-May-18	6	4	0 1	4	1.9	4	0.1
1-May-18	6	5	0 1	5	1.7	5	0.1
1-May-18	6	6	0 1	6	1.6	6	-0.1
4-May-18	9	7	0 1	7	1.6	7	0.1
4-May-18	9	8	0 1	8	1.9	8	0.1
4-May-18	9	9	0 1	9	1.7	9	0.1
7-May-18	12	10	0 1	10	2.0	10	0.1
7-May-18	12	11	0 1	11	1.6	11	0.1
7-May-18	12	12	0 1	12	2.0	12	0.1
11-May-18	16	13	0 1	13	1.8	13	0.1
11-May-18	16	14	0 1	14	2.0	14	0.1
11-May-18	16	15	0 1	15	1.9	15	0.1
15-May-18	20	16	0 1	16	2.0	16	0.1
15-May-18	20	17	0 1	17	2.0	17	0.1
15-May-18	20	18	0 1	18	2.0	18	0.1
18-May-18	23	19	0.0	19	2.2	19	0.0
18-May-18	23	20	0.0	20	2.1	20	0.0
18-May-18	23	21	0 1	21	2.1	21	0.1
21-May-18	26	22	0 1	22	1.7	22	0.1
21-May-18	26	23	0 1	23	2.0	23	0.1
21-May-18	26	24	0 1	24	1.7	24	0.1
23-May-18	28	25	0 1	25	1.9	25	0.1
23-May-18	28	26	0 1	26	2.1	26	0.1
23-May-18	28	27	0 1	27	1.9	27	0.1
23-May-18	28	28	0 1	28	1.8	28	0.1
23-May-18	28	29	0 1	29	2.1	29	0.2

[1]The total mass of inorganic carbon (IC) was calculated using the following equation:

TIC = (mg IC/L in vessel – mg IC/L added from 7M NaOH) × volume of liquid in vessel

[2]Calculations were performed in Excel 2010 full precision mode. Manual calculations may differ. ”Result” values were rounded for presentation purpose.

TABLE 5 PERCENT DEGRADATION[1,2]

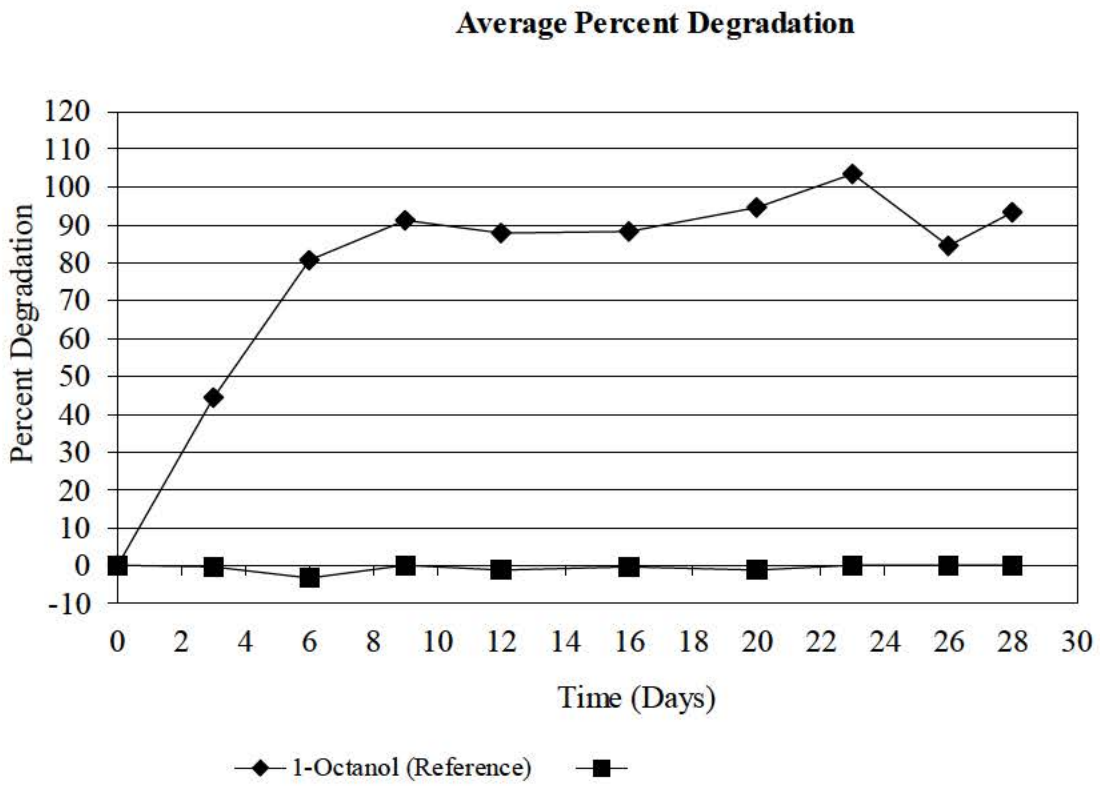
Date	Day	Control Replicate #	Result	1-Octanol Replicate #	Result	Replicate #	Result
28-Apr-18	3	1	NA	1	36.8	1	-0.6
28-Apr-18	3	2	NA	2	47.5	2	-1.2
28-Apr-18	3	3	NA	3	49.3	3	0.5
1-May-18	6	4	NA	4	88.1	4	-0.3
1-May-18	6	5	NA	5	80.7	5	-0.6
1-May-18	6	6	NA	6	73.3	6	-9.3
4-May-18	9	7	NA	7	77.6	7	-0.1
4-May-18	9	8	NA	8	90.8	8	0.2
4-May-18	9	9	NA	9	80.0	9	0.6
7-May-18	12	10	NA	10	94.4	10	-1.6
7-May-18	12	11	NA	11	72.8	11	-1.5
7-May-18	12	12	NA	12	95.8	12	0.3
11-May-18	16	13	NA	13	83.1	13	-0.3
11-May-18	16	14	NA	14	93.2	14	-0.6
11-May-18	16	15	NA	15	88.8	15	0.5
15-May-18	20	16	NA	16	93.1	16	-1.0
15-May-18	20	17	NA	17	96.1	17	-1.8
15-May-18	20	18	NA	18	94.8	18	0.2
18-May-18	23	19	NA	19	106.2	19	-0.5
18-May-18	23	20	NA	20	102.7	20	-0.4
18-May-18	23	21	NA	21	101.5	21	0.9
21-May-18	26	22	NA	22	81.6	22	0.4
21-May-18	26	23	NA	23	92.3	23	-0.7
21-May-18	26	24	NA	24	80.0	24	0.5
23-May-18	28	25	NA	25	92.7	25	-0.4
23-May-18	28	26	NA	26	98.7	26	-0.2
23-May-18	28	27	NA	27	89.9	27	-1.0
23-May-18	28	28	NA	28	86.2	28	-0.5
23-May-18	28	29	NA	29	100.2	29	3.7
Test Termination Average (Day 28)			NA		93.5		0.3
Standard Deviation					5.9		1.9

[1] The percent degradation at each sampling interval (Dt) was calculated using the following equation: Dt = [TICt – TICB] / TOC × 100 where TIC = total inorganic carbon and TOC = total organic carbon

TICt = mg TIC in test chamber at time t TICB = mean mg TIC in blank controls at time t TOC = mg TOC added initially to the test chamber

[2] Calculations were performed in Excel 2010 full precision mode. Manual calculations may differ. NA – Not Applicable.

FIGURE 1 AVERAGE PERCENT DEGRADATION



APPENDIX 1 CERTIFICATES OF ANALYSIS

APPENDIX 1 CERTIFICATES OF ANALYSIS (CONTINUED)

Reference Substance

SIGMA-ALDRICH®

3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	1-OCTANOL analytical standard, >= 99.7 % GC
Product Number:	95446
Batch Number:	BCBT3395
Brand:	Sigma-Aldrich
CAS Number:	111-87-5
Formula:	$CH_3(CH_2)_7OH$
Formula Weight:	130.23
Expiration Date:	NOV 2019
Quality Release Date:	13 DEC 2016

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	COLORLESS	COLORLESS
APPEARANCE (FORM)	LIQUID	LIQUID
PURITY (GC AREA %)	≥ 99.7 %	99.7 %
REMARKS ON GC	--	0.04 % 3-OCTANOL
REFRACTIVE INDEX N20/D	1.428 - 1.430	1.429
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

Dr. Claudia Geitner
Manager Quality Control
Buchs, Switzerland

APPENDIX 2. CHANGES TO THE STUDY PROTOCOL

This study was conducted in accordance with the protocol, with the following exceptions:

1. On page 6 the following was changed:

   Remove: Replicate chambers from each group will be removed for IC analysis twice every week over the test period. Three replicate test chambers from each group will be analysed at each interval.

   Add: Replicate chambers from each group will be removed for IC analysis twice every week over the test period. Three replicate test chambers from each group will be analysed at each interval except for end of study where 5 replicates from each group will be analyzed.

2. On page 2 the following was changed:

   Remove: Test Substance No. 14409 and Reference Substance No. 14242

   Add: Test Substance No 14633 and Reference Substance No. 14605

charles river

FINAL REPORT

Test Facility Study No. 20146045

Sponsor Reference No.

Weight of Evidence for Skin Sensitization of

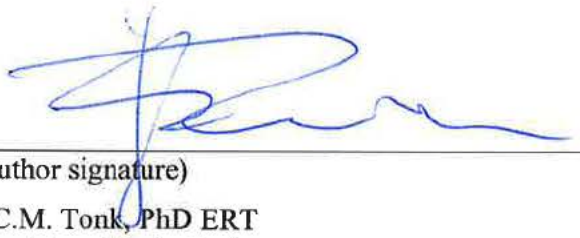
SPONSOR:

TEST FACILITY:
Charles River Laboratories Den Bosch BV
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

TABLE OF CONTENTS

AUTHENTICATION STATEMENT

We confirm that the information used in this statement is in accordance with the information provided by the sponsor and/or available from the studies performed. This information represents the current understanding and knowledge of the substance. If more (detailed) information becomes available or if updated guidance becomes available, the statement might be subject to review.



25-Sept.-2018

(Author signature) (Date)

E.C.M. Tonk, PhD ERT
Regulatory Toxicologist
Charles River Laboratories Den Bosch BV

i.a. S. Pelgrom

25-Sept-2018

(Reviewer signature) (Date)

H.M. Barentsen, PhD
Senior Regulatory Toxicologist
Charles River Laboratories Den Bosch BV

1. RESPONSIBLE PERSONNEL

1.1. Test Facility

Test Facility	Charles River Laboratories Den Bosch BV Hambakenwetering 7 5231 DD ‘s-Hertogenbosch The Netherlands

2. SUMMARY

The objective of this study was to reach an overall conclusion on the endpoint skin sensitization based on all available relevant information, including alternative testing data.

A DEREK assessment and DPRA assay were performed in accordance with Section 8.3 of Annex VII of Regulation (EC) No 1907/2006 as amended in Commission Regulation (EU) 2016/1688 of 20 September 2016 and the strategy presented in ECHA Guidance on information requirements and chemical safety assessment Chapter R.7a.

A reliable KeratinoSens™ study could not be performed, due to solubility issues with the substance under the experimental conditions of the test. The negative result observed in the DPRA assay is indicative for absence of skin sensitizing potential. Based on these results, no overall conclusion on skin sensitizing potential can be drawn, as no individual *in vitro* test is considered reliable to predict skin sensitizing properties of a substance. Based on a weight of evidence from the studies performed and the lack of conclusive information on skin sensitizing potential and/or potency, additional data is required to conclusively determine the classification.

3. INTRODUCTION

The objective of this study was to evaluate whether sufficient information is available to meet the information requirements for skin sensitization of Section 8.3 of Annex VII of Regulation (EC) No 1907/2006 as amended in Commission Regulation (EU) 2016/1688 of 20 September 2016 and the relevant classification in accordance with Regulation (EC) No 1272/2008 (CLP) and related amendments. A weight of evidence approach according to Annex XI, sections 1.2-1.5, to the REACH Regulation is used.

The design of this study is based on the following ECHA guidance:

Guidance on information requirements and chemical safety assessment Chapter R.7a Endpoint specific guidance v.6.0 July 2017, paragraph 7.3.

4. BACKGROUND/SCOPE

The weight of evidence approach is based on *in silico*/*in chemico*/*in vitro* data, addressing each of the following key events of skin sensitization on its own or together:

1. Key event 1: Covalent binding of the electrophilic substance to proteins; tested by OECD 442C: Direct Peptide Reactivity Assay (DPRA)
2. Key event 2: Release of pro-inflammatory cytokines and induction of cyto-protective pathways in keratinocytes; tested by OECD 442D: ARE-Nrf2 Luciferase Test Method or KeratinoSens™ assay
3. Key event 3: Activation and maturation of dendritic cells; OECD 442E Myeloid U937 Skin Sensitization Test (U-SENS™)
4. Key event 4: Presentation of the chemical allergen by dendritic cells to naïve T-cells, which leads to their differentiation and proliferation into allergen-specific memory T-cells; no generally accepted *in vitro* test available yet.

If information from test method(s) addressing one or two of these key events allows classification and risk assessment according to point 8.3 of Annex VII of the REACH Regulation, studies addressing the other key event(s) need not be conducted.
According to the Guidance on information requirements and chemical safety assessment R7a (v.6.0 July 2017), to reach a conclusion on (non-)classification, the following questions should be addressed:

- Does the evidence enable to conclude that the substance is not a skin sensitizer? If so, conclude on no classification.
- Does the evidence enable to conclude that the substance is presumed to produce significant sensitization in humans i.e. Cat. 1A? If so, classify accordingly.
- Does the evidence enable to conclude that the substance is a skin sensitizer and significant sensitization in humans i.e. Cat. 1A **can** be excluded? If so, it is presumed that the substance would be a moderate skin sensitizer i.e. Cat. 1B and it is recommended to classify accordingly.

In case none of these conditions are met, e.g. when Cat. 1A **cannot** be excluded, further testing needs to be performed, *in vivo* testing being the last resort. At the moment no accepted *in vitro* studies are available to discern between Cat. 1A and 1B.
In case of positive *in chemico*/*in vitro* skin sensitization tests and absence of reliable indication for potency by DEREK, for the time being performance of an *in vivo* study is the only option to determine the degree of potency (see CLP Regulation 3.4 Respiratory or skin sensitisation).

5. MATERIALS AND METHODS

5.1. Test Item

Identification:

Chemical name	
CAS number	
Purity/Composition	99.09%
UVCB	No

5.2. Method

As the substance is a mono-constituent and not a UVCB or metal the following strategy was followed:

STEP 0: The starting point for the weight of evidence is the assessment whether new studies are required. Available information on the test item was used to evaluate whether:

- The substance is not a strong acid (pH≤ 2.0) or base (pH ≥ 11.5), known to be not corrosive to the skin or (spontaneously) flammable in air or in contact with water or moisture at room temperature.
- No adequate existing human data, which provide evidence that the substance is a skin sensitizer are available.
- No data from existing studies on skin sensitization in laboratory animals, which provide sound conclusive evidence that the substance is a sensitizer or non-sensitizer are available.

This step is the responsibility of the sponsor.

If no reliable data on solubility is available, a solubility test is performed at Charles River to determine whether the substance dissolves sufficiently in a solvent which is appropriate for each test mentioned below. In case the solubility test demonstrates solubility of the test substance that meets the precondition limits for the *in vitro* tests, the following step-wise testing approach is followed:

STEP 1: DEREK assessment (overall skin sensitizing events), Direct Peptide Reactivity Assay (DPRA; molecular interaction with skin proteins) and KeratinoSens™ assay (inflammatory response in keratinocytes) are performed.

STEP 2: Depending on the outcome of the studies performed in STEP 1 and in absence of a definite conclusion on possible skin sensitization, the U-SENS™ assay is performed (key event: activation of dendritic cells).

STEP 3: Based on a weight of evidence of all available data on the test item related to skin sensitization, an argument is prepared to conclude on the classification for the substance or, if no conclusion can be drawn, to conclude on the performance of an *in vivo* skin sensitization study.

6. RETENTION OF RECORDS

The final report generated by Charles River from this study will be transferred to a Charles River archive no later than the date of final report issue.

7. RESULTS

No data were available that would preclude performance of the studies to determine the potential for skin sensitization. Therefore, STEP 1 studies were performed, i.e. DEREK assessment (project no 20146044), DPRA assay (project no 20146046) and KeratinoSens™ assay (project no 20146047).

7.1. Results of studies performed

DEREK NEXUS version 6.0.1 did not match the query structure with any structural alerts or examples for skin sensitization. However, the query structure contains features that were not found in the Lhasa skin sensitization negative prediction dataset (unclassified). is predicted to be not sensitizing to the skin, but this prediction should be considered with caution.

A valid DPRA test was performed according to OECD TG 442C and GLP principles. For the DPRA assay was dissolved in isopropanol at 100 mM. No co-elution of the test item with SPCC or SPCL was observed. In the cysteine reactivity assay the test item showed 3.1% SPCC depletion while in the lysine reactivity assay the test item showed 0.3% SPCL depletion. The mean of the SPCC and SPCL depletion was 1.7%. was negative in the DPRA and was classified in the "no or minimal reactivity class" when using the Cysteine 1:10 / Lysine 1:50 prediction model. However, since precipitation was observed upon preparation and after the incubation period for SPCL, one cannot be sure how much test item remained in the solution to react with the peptides. In this case with only a precipitate observed for the lysine assay, the Cysteine 1:10 prediction model may be used and the DPRA still results in a negative prediction.

A KeratinoSens™ assay could not be performed due to solubility issues. is not soluble in the standard solvents dimethyl sulfoxide, Milli-Q water and ethanol. In addition, a homogenous suspension could not be obtained in these solvents. Therefore, it was concluded that the KeratinoSens™ assay is not suitable for this test item.

8. DISCUSSION

Based on a negative DEREK NEXUS assessment and a negative DPRA assay, no conclusion can be drawn on skin sensitization of The negative result observed in the DPRA assay is indicative for the absence of skin sensitizing potential of A reliable KeratinoSens™ assay could not be performed, due to solubility issues with the substance under the experimental conditions of the test. Performance of a third *in vitro* assay, the U-SENS™ assay, according to STEP 2 would not yield additional information as this assay uses the same solvents and similar solubility issues are to be expected as observed for the KeratinoSens™ assay.

No overall conclusion on skin sensitizing potential can be drawn, as no individual *in vitro* test is considered reliable to predict skin sensitizing properties of a substance. Based on a weight of evidence from the studies performed and the lack of conclusive information on skin sensitizing potential and/or potency, additional data is required to conclusively determine the classification.

9. CONCLUSION

Based on the *in vitro* tests performed no definite conclusion on skin sensitizing potential and/or potency can be drawn for In the absence of a reliable and for registration purposes accepted non-*in vivo* test, additional data is required to conclusively determine the skin sensitizing properties of the substance.

10. REFERENCES

S. Jonis, DEREK Prediction on Skin Sensitization of
Charles River Laboratories Den Bosch BV, The Netherlands, project no 20146044, 28 March 2018.

J. Reinen, *In Chemico* Determination of the Skin Sensitization Potential of
using the Direct Peptide Reactivity Assay (DPRA), Charles River Laboratories Den Bosch BV, The Netherlands, project no 20146046, 14 Jun 2018.

J.A.J. Woutersen, Evaluation of the in vitro Skin Sensitization Potential of
with the KeratinoSens™ assay, Charles River Laboratories Den Bosch BV, The Netherlands, project no 20146047, 29 Jun 2018.

FINAL REPORT

Study Title

SKIN IRRITATION TEST (SIT) USING THE EPIDERM™ SKIN MODEL

Test Article

Test Guideline

OECD Guideline 439 (2015)

Authors

Gertrude-Emilia Costin, Ph.D., M.B.A.
Kayla Campasino, M.S.

Study Completion Date

8 August 2018

Performing Laboratory

Institute for In Vitro Sciences, Inc.
30 W. Watkins Mill Road, Suite 100
Gaithersburg, MD 20878

Study Number

18AD37.050082

Laboratory Project Number

9835

Sponsor Study Number

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STATEMENT OF COMPLIANCE

Skin Irritation Test (SIT) Using The Epiderm™ Skin Model of the test article, was conducted in compliance with the U.S. EPA (TSCA) GLP Standards 40 CFR 792 and the principles presented in the OECD series on Good Laboratory Practice, in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the assay controls has not been determined by the testing facility.

The Certificates of Analysis for the assay controls provided by their respective manufacturers and for the test article provided by the Sponsor are included in Appendix C.

The stability of the test article and the controls under the storage conditions at the testing facility and under the actual test conditions has not been determined by the testing facility and is not included in the final report.

Gertrude-Emilia Costin, Ph.D., M.B.A.
Study Director

8 August 2018
Date

QUALITY ASSURANCE STATEMENT

Study Title: Skin Irritation Test (SIT) Using the EpiDerm™ Skin Model

Study Number: 18AD37.050082

Study Director: Gertrude-Emilia Costin, Ph.D., M.B.A.

This study was divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitored each of these phases over a series of studies. Procedures, documentation, equipment records, etc., were examined in order to assure that the study was performed in accordance with the U.S. EPA (TSCA 40 CFR 792) GLP Standards, which agree in principle with the OECD Principles of Good Laboratory Practice and to assure that the study was conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director and Management
Protocol and Initial Paperwork	17-May-18	17-May-18
Rinsing of tissues	23-May-18	23-May-18
Final Report	30-Jul-18 31-Jul-18	31-Jul-18

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Megan Conahan — 31 Jul 2018

Megan Conahan, B.S.
Quality Assurance

Date

SIGNATURE PAGE

SKIN IRRITATION TEST (SIT)
USING THE EPIDERM™ SKIN MODEL

Initiation Date:	17 May 2018
Completion Date:	8 August 2018
Sponsor:	
Sponsor's Representative:	
Testing Facility:	Institute for In Vitro Sciences, Inc. 30 W. Watkins Mill Road, Suite 100 Gaithersburg, MD 20878
Archive Location:	Institute for In Vitro Sciences, Inc. Gaithersburg, MD 20878
Study Director:	Gertrude-Emilia Costin, Ph.D., M.B.A. — 8 August 2018 (Date)
Director of Laboratory Operations:	Greg Mun, B.A.

TEST ARTICLE RECEIPT

IIVS Test Article Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions*
18AD37		clear colorless non-viscous liquid	6 April 2018	room temperature

* - Protected from exposure to light

SKIN IRRITATION TEST (SIT)
USING THE EPIDERM™ SKIN MODEL

INTRODUCTION

The purpose of this study was to evaluate the skin irritation potential of the test article, supplied by in the context of identification and classification of skin irritation hazard according to the UN GHS and EU classification system (Category 2/Category 1 and No Category). The skin irritation potential was evaluated based upon measuring the relative conversion of MTT (3-[4,5 - dimethylthiazol-2-yl] - 2,5 - diphenyltetrazolium bromide)[1] in the test article-treated tissues after exposure to the test article for a 60-minute exposure period, followed by a 42-hour post-exposure expression period. Skin irritation potential of the test article was predicted if the relative viability was less than or equal to 50%. This study was conducted according to the OECD guideline 439, "*In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method", adopted 28 July 2015[2].

The test article was tested in one valid definitive assay to determine the identification and classification of skin irritation hazard according to the UN GHS classification system (Category 1/2 or No Category). The laboratory phase of the study was conducted from 21 May 2018 to 25 May 2018 at the Institute for In Vitro Sciences, Inc.

[1] Berridge, M.V., Tan, A.S., McCoy, K.D., Wang, R. (1996) The Biochemical and Cellular Basis of Cell Proliferation Assays That Use Tetrazolium Salts. Biochemica 4:14-19.

[2] OECD Guidelines for the Testing of Chemicals. *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method (OECD TG 439), adopted 28 July 2015.

MATERIALS AND METHODS

Receipt of the EpiDerm™ Skin Model

Upon receipt of the EpiDerm™ Skin Kit (MatTek Corporation), the solutions were stored as indicated by the manufacturer. The EpiDerm™ tissues were stored at 2-8ºC until use. On the day prior to testing, EpiDerm™ Maintenance Medium was set to room temperature prior to use. Nine-tenths mL of Maintenance Medium were aliquoted into the appropriate wells of 6-well plates. Each 6-well plate was labeled with the test article, positive control, or negative control. Each EpiDerm™ tissue was inspected for air bubbles between the agarose gel and cell culture insert prior to opening the sealed package. Tissue inserts with air bubbles covering greater than 50% of the cell culture insert area were not used. The 24-well shipping containers were removed from the plastic bag and their surfaces were disinfected with 70% ethanol. The EpiDerm™ tissues were transferred aseptically into the 6-well plates. The EpiDerm™ tissues were then incubated at 37±1ºC in a humidified atmosphere of 5±1% CO_2 in air (standard culture conditions) for 60±5 minutes. After 60±5 minutes, the EpiDerm™ tissues were transferred to appropriate wells containing 0.9 mL of fresh warmed (to 37ºC) Maintenance Medium. The plates were returned to the incubator for 18±3 hours to acclimate the tissues.

Test Article Preparation

As instructed by the Sponsor, the test article was administered to the test system without dilution. The test article was handled in a fully vented hood. The test article was provided in a liquid nitrogen pressurized cylinder. Following the instructions provided by the Sponsor (see Protocol Attachment 1), an aliquot needed for the assay (~1.0 mL) was removed from the cylinder and placed into a glass vial, which was stored at room temperature. The tissues treated with the test article were cultured on separate plates from the assay controls.

Assessment of Test Article/Nylon Mesh Compatibility

The test article, was not observed to interact with the nylon mesh, and therefore a nylon mesh was used to aid in the spreading of the test article after dosing the EpiDerm™ tissues.

Assessment of Direct Test Article Reduction of MTT

The test article was added to a 1.0 mg/mL MTT (Sigma) solution in warm Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine (MTT Addition Medium) to assess its ability to directly reduce MTT. Approximately one leveled spoonful (approximately 25 mg) (solid test articles) of the test article were added to 1 mL of the MTT solution and the mixtures were incubated in the dark at standard culture conditions for at least one hour. A negative control, 30 µL of sterile, Calcium and Magnesium Free Dulbecco's Phosphate Buffered Saline (CMF-DPBS), was tested concurrently. If the MTT solution color turned blue/purple, the test article was presumed to have reduced the MTT.

The test article was not observed to directly reduce MTT in the absence of viable cells.

Assessment of Colored or Staining Materials

Prior to conducting any assays with viable tissues, the ability of the test article to interfere with the photometric MTT measurement was assessed. The test article was checked for its colorant properties (*i.e.,* their ability to absorb light significantly at the wavelength used for the MTT determination. Approximately 30 µL (liquid test articles) were added to 2.0 mL isopropanol in 6-well plates and placed on an orbital place shaker for 2-3 hours at room temperature. After shaking, 200 µL aliquots of the isopropanol solutions and two blank samples of isopropanol were transferred to a 96-well plate and the absorbance was measured with a plate reader at the MTT measurement wavelength (570 nm). The absorbance of the test article samples was determined by subtracting the mean isopropanol blank value from the absorbance of the test article samples. If the OD_{570} of the test article sample was > 0.08, the material has to be considered as possibly interacting with the MTT measurement.

The test article was not considered to have probable photometric MTT interference.

pH Determination

The pH of the test article was measured using pH paper (EMD Millipore Corporation). Initially, the test article was added to pH paper with a 0-14 pH range in 1.0 pH unit increments to approximate a narrow pH range. Next, the test article was added to pH paper with a narrower range of 0-6 pH units with 0.5 pH unit increments, to obtain a more accurate pH value. The pH value obtained from the narrower range pH paper is presented in Table 1.

Controls

The definitive assay included a negative control and a positive control. The negative control was 30 µL of sterile, CMF-DPBS and the positive control was 30 µL of 5% Sodium Dodecyl Sulfate (SDS). Both the positive and negative controls were tested in triplicate, and at the same exposure time as the test article (60±1 minutes).

Skin Irritation Test (SIT) Definitive Assay

The test article was tested in one valid definitive trial.

After the overnight incubation of 18±3 hours, the 6-well plates containing the EpiDerm™ tissues were removed from the incubator and placed at room temperature for at least 5 minutes prior to dosing.

The EpiDerm™ tissues were treated in triplicate with the test article for 60±1 minutes. Since the test article was a powder, immediately before its application, each tissue surface was moistened with 25 µL of sterile CMF-DPBS to improve contact of the tissue surface with the test chemical. After adding the CMF-DPBS, 25 mg of the test article were added to each corresponding three tissues at 1 minute intervals per tissue using a 25 mg sharp spoon (Aesculap #FK 623R). The sharp spoon was filled with the test article and then the spoon was leveled. After the three tissues were dosed with the test article, the test article was gently mixed and

spread over the tissue surface using a sterile bulb-headed rod. The EpiDerm™ tissues were tested in triplicate with the positive and negative control, respectively, for 60±1 minutes. Thirty microliters of each control were applied to each of three tissues at 1 minute intervals per tissue. Immediately after control administration onto the tissue, a nylon mesh was placed gently over the dose to spread the negative and positive controls. The plates with dosed tissues were kept in the laminar flow hood until the last tissue was dosed. After the last tissue was dosed, all of the plates were transferred to the incubator for 35±1 minutes at standard culture conditions. After 35±1 minutes, all of the plates were removed from the incubator, placed into the laminar flow hood and kept at room temperature until the exposure period was completed for the first dosed tissue.

After 60±1 minutes of control or test article exposure, the tissues were rinsed with sterile, CMF-DPBS by filling and emptying the tissue insert 15 times. A stream of CMF-DPBS was directed onto the tissue surface. The mesh used to spread the test article and control doses was carefully removed with forceps (if necessary) after the 5th rinse. After the removal of the mesh, the rinsing procedure of the tissue continued for 10 times. After the 15th rinse, each of the 3 inserts per treatment group (test article, positive and negative control) was completely submerged, gently swirled, and rinse media dumped in a beaker containing approximately 150 mL of CMF-DPBS and specifically assigned for each treatment group; this procedure was repeated three times for each insert of each treatment group. Finally, the tissues were rinsed once more on the inside and outside of the tissue insert with sterile CMF-DPBS from the wash bottle, and the excess CMF-DPBS was decanted. The bottoms of the tissue inserts were blotted on sterile paper towels and the inserts were transferred to new 6-well plates containing 0.9 mL of fresh warmed (to 37ºC) Maintenance Medium. The tissue surface was carefully blotted with sterile cotton-tipped applicators to remove any excess moisture, and the tissue surface was visually observed for residual test article using a dissecting scope. The tissues were then placed into the incubator at standard culture conditions for a post-treatment expression incubation of 42±2 hours. After an initial 24±1 hours of incubation, the 6-well plates were removed from the incubator and the tissues were transferred into new 6-well plates pre-filled with 0.9 mL fresh Maintenance Medium warmed to approximately 37ºC. The tissues were placed back into the incubator at standard culture conditions for an additional 18±1 hours for the remainder of the 42±2 hour post-treatment expression incubation.

MTT Preparation

A 10X stock of MTT prepared in PBS (filtered at time of batch preparation) was thawed and diluted in warm MTT Addition Medium to produce a 1.0 mg/mL solution no more than two hours before use. Three hundred microliters of the MTT solution were added to each designated well of a pre-labeled 24-well plate.

After the total 42 ± 2 hours post-exposure expression incubation, the 6-well plates were removed from the incubator. Each tissue was blotted on a sterile paper towel and transferred to an appropriate well containing 0.3 mL of MTT solution. The 24-well MTT plates were incubated at standard culture conditions for 3±0.1 hours.

After the 3±0.1 hours incubation, the EpiDerm™ tissues were submerged, gently swirled, and rinse media decanted in a beaker containing approximately 150 mL of CMF-DPBS three times. The tissue was then blotted on absorbent paper, cleared of excess liquid, and transferred to a pre-labeled 24-well plate containing 2.0 mL of isopropanol in each designated well. The plate was covered with parafilm and shaken for 2 - 3 hours at room temperature to extract the MTT. At the end of the extraction period, the insert was gently agitated up and down in its extractant well. The tissues were pierced with forceps to allow the extract to flow back into the well from which the insert was removed, and the cell culture inserts were discarded. The extract solution was mixed (homogenized by pipetting up and down three times) and two x 200 µL aliquots were transferred to the appropriate wells of a 96-well plate. Two x 200 hundred µL of isopropanol were added to the wells designated as blanks. The absorbance at 570 nm (OD_{570}) of each well was measured with a Molecular Devices Vmax plate reader with the AUTOMIX function selected.

Presentation of Data

The mean OD_{570} value of the blank wells was calculated. Individual blank-corrected OD_{570} values for each of the duplicate aliquots of the test article or control tissue was determined by subtracting the mean OD_{570} value of the blank wells from their individual OD_{570} values. All calculations were performed using an Excel spreadsheet.

$$\text{Corrected Individual } OD_{570} = \text{Individual } OD_{570} - \text{mean Blank } OD_{570}$$

Mean corrected OD_{570} values were calculated for each individual test article and control tissue from the duplicate aliquots. The group mean of the corrected OD_{570} values for the negative controls were calculated.

$$\% \text{ Viability} = \frac{\text{Mean Corrected } OD_{570} \text{ of Aliquots of Individual Test Article or Control Tissue}}{\text{Corrected Group Mean } OD_{570} \text{ of Negative Control}} \text{ X } 100$$

The individual % of Control viability values were tabulated for each individual tissue. Mean (and standard deviation) viability values were calculated for the test article and control treatment groups. Finally, the mean viability values were plotted on a bar graph (with 1 standard deviation error bar) for the test article and positive control.

Evaluation of Test Results

The following Prediction Model was endorsed by the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) for the prediction of skin irritation. A test article was predicted to be an irritant (GHS Category 1 or 2) when the mean relative viability of the three treated tissues is less than or equal to 50% of the mean viability of the negative control.

In Vitro Result	*In Vivo* Prediction	GHS Category
mean tissue viability ≤ 50%	Irritant (I)	Category 1 or 2*
mean tissue viability > 50%	Non-irritant (NI)	No Category

**- to discriminate between GHS Category 1 and 2, additional testing required*

Criteria for a Valid Test

The assay was accepted when the following criteria were met: 1) the positive control (5% SDS) resulted in a mean tissue viability ≤ 20%, 2) the mean OD_{570} value of the negative control tissues was ≥ 0.8 and < 2.8, and 3) the standard deviations of the positive and negative control calculated from individual percent tissue viabilities of the three identically treated replicates were < 18%.

RESULTS AND DISCUSSION

The test article was tested using the EpiDerm™ Skin Model for the Skin Irritation Test (SIT). Table 1 summarizes the results of the Skin Irritation Test (SIT) for the test article and the positive control. The raw and analyzed data for the test article and the negative and positive controls are included in Appendix B. The mean OD_{570} of the negative control, CMF-DPBS, was 1.384. The mean viability of the positive control, 5% SDS, was 4.12%. The standard deviation calculated from individual percent tissue viabilities of the 3 identically treated replicates was <18% for the positive control and negative control. Since the acceptance criteria were met, the assay was considered valid.

The test article was not observed to directly reduce MTT in the absence of viable cells.

The test article was not determined to be a colorant (was not considered to have potential interference with the MTT measurement).

Based upon the results of this assay, the test article was predicted to be non-irritating to the skin, and thus does not to not require classification and labelling for skin irritation according to UN GHS and EU classification system (No Category).

SIT Results Using the EpiDerm™ Skin Model
Table 1

Assay Date	IIVS Test Article Number	Sponsor's Designation	Conc.	Mean Viability (%)	Skin Irritation Prediction[+]	pH
23 May 2018	18AD37		neat	112.6	Non-Irritant	5.0
	Positive Control	SDS	5% (w/v)	4.12	Irritant	NA

[+] - A test article was predicted to be a skin irritant (GHS Category 1 or 2) if the mean relative viability of the three treated tissues was ≤ 50%

NA – Not Applicable

APPENDIX A

SKIN IRRITATION TEST (SIT) USING THE EPIDERM™ SKIN MODEL

1.0 PURPOSE

The purpose of this study is to predict skin irritation potential of the test article using the EpiDerm™ reconstructed human epidermis model in the context of identification and classification of skin irritation hazard according to the United Nations (UN) Globally Harmonized System (GHS). Toxicity will be determined by measuring the relative conversion of MTT (3-[4,5 - dimethylthiazol-2-yl] - 2,5 - diphenyltetrazolium bromide) to a formazan product in the test article-treated tissues after exposure to the test article for a 60-minute exposure period, followed by a 42-hour post-exposure expression period. The protocol meets the requirements of the OECD guideline, "*In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method" (TG **439**).

2.0 SPONSOR

2.1 Name:

2.2 Address:

2.3 Representative:

2.4 Sponsor Study No.:

3.0 IDENTIFICATION OF TEST ARTICLE(S) AND ASSAY CONTROLS

3.1 Test Article(s): See Protocol Attachment I

3.2 Assay Controls:

Positive:	5% SDS (sodium dodecyl sulfate) [CAS# 151-21-3]
Negative:	Sterile, Ca^{++} and Mg^{++} Free Dulbecco's Phosphate Buffered Saline (CMF-DPBS)

3.3 Determination of Strength, Purity, etc.

3.3.1 For GLP studies, the Institute for In Vitro Sciences, Inc. (IIVS) will attempt to secure documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions from the Sponsor. If the Sponsor is unable to provide such information the final report will be generated with an exception noted in the Statement of Compliance.

3.3.2 IIVS will be responsible for the documentation of the analytical purity and composition of the negative control and the SDS used for the stock or working dilution of the positive control. This may be accomplished by maintaining a certificate of analysis from the supplier.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Institute for In Vitro Sciences, Inc.
4.2	Address:	30 W. Watkins Mill Road, Suite 100 Gaithersburg, MD 20878
4.3	Study Director:	Gertrude-Emilia Costin, Ph.D., M.B.A.

5.0 TEST SCHEDULE

5.1	Proposed Experimental Initiation Date:	21 May 2018
5.2	Proposed Experimental Completion Date:	8 June 2018
5.3	Proposed Report Date:	3 August 2018

6.0 TEST SYSTEM

The EpiDerm™ Model (EPI-200) (MatTek Corporation, Ashland, USA) consists of normal, human-derived epidermal keratinocytes which have been cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered *stratum corneum* containing intercellular lamellar lipid layers arranged in patterns analogous to those found *in vivo*. The EpiDerm™ Model incorporates several features which make it advantageous in the study of potential dermal toxicity. First, the test system uses a serum-free medium which eliminates the possibility of serum protein and test article interaction (Shopsis and Eng, 1988). Secondly, the target cells are epithelial, derived from human skin. Third, since the tissue has a functional *stratum corneum*, the test materials are applied directly to the tissue surface, at air interface, so that undiluted and/or end use dilutions can be tested directly. Prior to use, each 6, 24 and 96-well plate will be uniquely identified with a number written in permanent marker with the test article identification or control treatment group.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The experimental design of this study consists of the determination of the direct MTT reduction potential, assessment of colorant potential, a pH determination of the neat liquid test article and/or dosing dilution, if possible, and a definitive Skin Irritation Test (SIT). On the day of receipt, the EpiDerm™ tissues are conditioned by an overnight incubation for release of transport-stress related compounds and debris. After pre-incubation, tissues are topically exposed in triplicate to the test article(s), positive control, and negative control for 60 minutes. Tissues are then thoroughly rinsed to remove the test or control article, blotted, and transferred to fresh medium. After a 24 hour

incubation period, the tissues are refed with fresh medium, and incubated for another 18 hours for a total 42-hour post-exposure incubation period. Viability will be determined by the NAD(P)H-dependent microsomal enzyme reduction of MTT in control and test article-treated tissues (Berridge et al., 1996). The MTT assay is performed by transferring the tissues to 24-well plates containing MTT medium (1 mg/mL). After a 3-hour MTT incubation, the blue formazan salt formed by cellular mitochondria is extracted with 2 mL isopropanol per tissue and the optical density of the extracted formazan is determined with a spectrophotometer at 570 nm. Relative cell viability is calculated for each tissue as % of the mean of the negative control-treated tissues. Skin irritation potential of the test article(s) is predicted if the relative viability is less than or equal to 50%.

7.1 Media, Reagents and Supplies

7.1.1 EpiDerm™ Maintenance Medium: EPI-100-NMM Supplied by MatTek Corporation

7.1.2 EpiDerm™ Skin Model: EPI-200 supplied by MatTek Corporation

7.1.3 MTT (3-[4,5 - dimethylthiazol-2-yl] - 2,5 - diphenyltetrazolium bromide) (Sigma, or equivalent)

7.1.4 Ca^{++} and Mg^{++} Free Dulbecco's Phosphate Buffered Saline (CMF-DPBS) (Quality Biologicals, or equivalent)

7.1.5 Extraction Solution: Isopropanol (Sigma, or equivalent)

7.1.6 Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine (MTT Addition Medium) (Quality Biological, or equivalent)

7.1.7 Sharp spoon (for delivering approximately 25 mg of solids) Aesculap, Catalog No. FK 623R

7.1.8 Mesh (Nylon 150 µm, BUISINE ref: 03150/44, or similar): 8 mm discs for allowing liquids to spread over tissue surface

7.1.9 Cotton tip swabs (sterile): for blotting the tissue surface

7.2 Environmental Conditions

Throughout this protocol, ranges for test material and test system exposure or incubation conditions (*e.g.*, temperature, humidity, CO_2,) are presented. These ranges describe the equipment performance specifications under static conditions (*i.e.*, in the absence of frequent opening of equipment doors, accessing chambers, changing loads, etc.), as presented in the relevant equipment SOPs.

7.3 Preparation and Delivery of Test Article for the SIT

The test article will be prepared as directed by the Sponsor. Test articles will generally be tested neat. End use concentrations or other forms may be used as directed by the Sponsor. The exact test article preparation procedure and exposure conditions will be outlined in Protocol Attachment 1.

Thirty microliters (**30 µL**) of liquid test article or approximately 25 mg of a solid test article (measured using a level Aesculap FK 623R sharp spoon) will be delivered to the EpiDerm™ tissue.

7.3.1 Liquids: Thirty microliters (**30 µL**) of pipettable substances, such as liquids, gels, creams, and foams, will be applied directly on the tissue using a positive displacement pipet so as to cover the upper surface.

7.3.1.1 Fluid Liquids: Immediately after applying the fluid liquid test article, a circular nylon mesh with an 8 mm diameter will be placed onto each treated tissue. The nylon mesh is intended to encourage complete coverage of the liquid test articles over the tissue surface by capillary action.

7.3.1.2 Viscous Liquids/Gels/Pastes: **30 µL** will be dispensed directly atop the tissues. If necessary the chemical will be spread with Pasteur pipette to match size of tissue.

Viscous pipettable materials may first be transferred to a syringe to aid in filling a pipet. The pipet tip of the positive displacement pipet will be inserted into the dispensing tip of a syringe so that the test article can be loaded into the displacement tip under pressure. Simultaneously, the syringe plunger will be depressed as the pipet piston is drawn upwards. A dosing device (a flatheaded cylinder of slightly less diameter than the inner diameter of the cell culture insert) may be placed over the test article to assure even spreading, if needed.

Test articles which are too viscous to spread over the tissue will first be spread onto the flat end of a dosing device. The dosing device will be put into the cell culture insert to bring the test article in contact with the tissue. When the dosing device is used, approximately **30 µL** or 25±1 mg will be weighed and will be applied to the dosing device so as to cover the dosing surface. The sample should be spread to form a relatively smooth even layer on the surface of the dosing device to maximize uniform tissue contact. Semi-solids (*e.g.*, pastes, cream) can be pre-softened by transferring the test article to a syringe affixed with a three way stopcock attached to a second syringe. The sample will be pushed from syringe to syringe (i.e., creamed) until it is a pipetteable consistency.

7.3.2 Solids: Dry solids or powders may be ground with a mortar and pestle to improve consistency, if needed.

Shortly before application of the solid test article, the tissue surface will be moistened with 25 µL of sterile CMF-DPBS to improve contact with the test article. After adding the CMF-DPBS, the solid test article will be placed directly onto the moistened tissue at approximately 25 mg/tissue using a 25 mg sharp spoon. The sharp spoon will be filled with test article and leveled by gently stroking away excess test article. Care will be taken to avoid packing the material into the spoon. The contents of the spoon will be poured over the tissue surface. The test article will be gently mixed and spread over the tissue surface using a sterile bulb-headed rod.

The exact test article preparation procedure and exposure conditions used for other test article forms will be determined after consultation with the Sponsor and/or the Study Director (see Protocol Attachment 1). All exposure conditions will be documented in the study workbook.

The stability of the test article under the storage conditions at the testing facility and under the actual experimental conditions will not be determined by Institute for In Vitro Sciences, Inc. (IIVS).

7.4 Route of Administration

Test article will be administered by direct application to the surface of the EpiDerm™ tissues.

7.5 pH Determination

The pH of the neat liquid test article (and/or dosing dilution as appropriate) will be determined, if possible. The pH will be determined using pH paper (e.g., with a pH range of 0 – 14 to estimate, and/or a narrower pH range of 5 – 10). The typical pH increments on the narrower range pH paper are approximately 0.3 to 0.5 pH units. The maximum increment on the pH paper is 1.0 pH units.

7.6 Assay Controls

The Skin Irritation Test (SIT) will include a negative control and a positive control.

7.6.1 The negative control, **30 µL** of sterile CMF-DPBS, will be tested in triplicate tissues for a 60±1.0 minute exposure. The tissues will be handled exactly as described in §**7.9**.

7.6.2 The positive control, **30 µL** of 5% SDS, will be tested in triplicate tissues for a 60±1.0 minute exposure. The tissues will be handled exactly as described in §**7.9**.

7.7 Pre-Assay Test Article Compatibility Tests

7.7.1 Assessment of Direct Test Article Reduction of MTT

The ability of each test article to directly reduce MTT will be determined prior to testing in the definitive assay. A 1.0 mg/ml MTT solution will be prepared in warm MTT Addition Medium as described in **§7.10**. **Thirty (30) µL** (liquid test articles) or approximately 25 mg (solid test articles) will be added to 1 mL of the MTT solution and the mixture incubated in the dark at 37±1°C for one to three hours. A negative control, **30 µL** of sterile, CMF-DPBS, will be tested concurrently. If the MTT solution color turns blue/purple, the test article is presumed to have reduced the MTT. Water insoluble test materials may show direct reduction (darkening) only at the interface between the test article and the medium.

The MTT direct reduction test for the test article(s) may have been previously performed in an independent study. In such cases, the results of the MTT direct reduction test may be used for this specific study and the initial study will be referenced.

7.7.2 Nylon Mesh Compatibility Test

Since some chemicals may react with the nylon mesh, the compatibility of each liquid test article with the nylon mesh should be checked. To test if a test article interacts with the mesh, the mesh is placed on a slide and **30 µL** of test article are applied on it. A negative control, **30 µL** of sterile, CMF-DPBS, will be tested concurrently. The slides will be left covered in a petri dish and incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air (standard culture conditions) during the 60±1 minute exposure. After the 60±1 minute exposure, the mesh is observed microscopically. If an interaction between the test article and the mesh is observed, test article will be applied without using a mesh as a spreading aid.

7.7.3 Assessment of Colored or Staining Materials

Prior to conducting any assays with viable tissues, the test article(s) ability to interfere with the photometric MTT measurement will be assessed. Each test article should be checked for its colorant properties (*e.g.*, their ability to absorb light significantly at the wavelength used for the MTT determination).

Approximately 30 µL (liquid test articles) or one leveled spoonful (approximately 25 mg) (solid test articles) will be added to 2.0 mL isopropanol in 6-well plates and placed on an orbital plate shaker for 2-3 hours at room temperature. After shaking, 200 µL aliquots of the isopropanol solutions and two blank samples of isopropanol will be transferred to a 96-well plate and the absorbance will be measured with a plate reader at the MTT measurement wavelength (570 nm).

Some test articles may precipitate or become cloudy after addition to the isopropanol and interfere with the optical density (OD) measurement. In such cases, after the 2-3 hour shaking period, the test article-isopropanol mixture may be transferred into centrifuge tubes and centrifuged (up to 14,000 rpm, for up to 5 minutes at room temperature) prior to transfer to the 96-well plates for the absorbance determination. If the mixture is cloudy and/or precipitate is noted after the initial plate reading, the sample may be centrifuged after the initial plate reading, added to the 96-well plate, and then the plate re-read. The same centrifugation procedure may be performed after the isopropanol extraction period in the definitive assay.

The absorbance of the test article samples will be determined by subtracting the mean isopropanol blank value from the absorbance of the test article samples. If the OD_{570} of the test article sample is **> 0.08** the test article will be treated as outlined in section §7.12 for the colorant controls procedures.

7.8 Receipt of EpiDerm™

Upon receipt of the EpiDerm™ Kit, the reagents will be stored as indicated by the manufacturer. The EpiDerm™ tissues will be stored at 2-8°C until used.

On the day prior to testing, an appropriate volume of EpiDerm™ Maintenance Medium will be removed and equilibrated to room temperature. Nine-tenths mL of Maintenance Medium will be aliquoted into 6-well plates. One 6-well plate may be used for pre-incubation of three tissues. Each EpiDerm™ tissue will be inspected for air bubbles between the agarose gel and cell culture insert prior to opening the sealed package. Cultures with air bubbles greater than 50% of the cell culture insert area will not be used. The 24-well shipping containers will be removed from the plastic bag and the surface disinfected by wiping with absorbent paper moistened with 70% ethanol. An appropriate number of EpiDerm™ tissues will be aseptically removed from the 24-well shipping plate and transferred to the 6-well plates containing Maintenance Medium. **Excess moisture may be absorbed from the tissue surface using a sterile cotton tip swab**. The EpiDerm™ tissues will be incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air (standard culture conditions) for 60 ± 5 min. At the end of the first pre-incubation period, the tissues will be transferred into wells containing fresh pre-warmed (to 37±1°C) Maintenance Medium (for example, from the upper wells into the lower wells of the 6-well plate which may be pre-filled) and will be incubated overnight (18 ± 3 hrs) at standard culture conditions to acclimate the tissue.

7.9 Skin Irritation Test Definitive Assay

A set of tissues should be removed from the incubator and moved to room temperature **(19 to 28 °C)** in the laminar flow hood at least 5 minutes before beginning the test or control article treatments. The temperature in the laminar

flow hood where the tissues will be dosed will be recorded within ± 2 hours of test article dosing. Thirty microliters (**30 µL**) of each liquid test article, negative or positive control, or approximately 25 mg of each solid test article (using an Aesculap 25 mg sharp spoon) will be applied to each of three tissues at 1 minute intervals per tissue. The plates with dosed tissues will be kept in the laminar flow hood until the last tissue of the set is dosed. After dosing, the plates will be transferred to the incubator and incubated for 35± 1 minutes at standard culture conditions. After 35± 1 minutes, all the plates will be removed from the incubator, placed into the laminar flow hood and kept there until the exposure period of 60± 1 minutes has been completed for the first dosed tissue.

After 60± 1 minutes of test or control article exposure, the tissues will be rinsed with sterile CMF-DPBS, filling and emptying the cell culture insert **approximately 15 times** to remove any residual test article (Figure 1). **A stream of CMF-DPBS will be directed onto the tissue surface to assure removal of the test or control article.**

For test and control articles where a mesh was used, **the mesh will be carefully removed with forceps (if necessary)**. The mesh will be removed when it dislodges from the tissues or after the fifth rinse (whichever comes first). After the removal of the mesh, the rinsing procedure of the tissue will continue for the remaining rinses (total of 15 rinses).

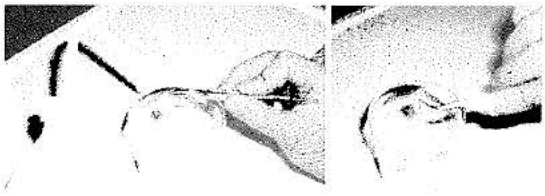


Figure 1: fill insert with CMF-DPBS & decant CMF-DPBS

After the 15th rinse, the cell culture inserts will be completely submerged, gently swirled, and rinse media dumped in a beaker containing approximately 150 mL of CMF-DPBS. This process will be repeated three times. Finally, the tissues will be rinsed once more on the inside and outside of the cell culture insert with sterile CMF-DPBS from the wash bottle, and the excess CMF-DPBS will be decanted.

The cell culture insert bottom will be blotted on sterile paper towels (Figure 2). The cell culture inserts will be transferred to new 6-well plates containing 0.9 mL/well of fresh pre-warmed Maintenance Medium pre-warmed to 37±1°C. The surface of each tissue will be carefully dried with a sterile cotton tipped swab (Figure 3).

Figure 2: blotting

Figure 3: drying tissue surface

The tissue surface will be evaluated for test article residues using a dissecting stereoscope. If test article residues are observed, attempts may be made to remove the residues using a sterile cotton swab (pre-moistened with CMF-DPBS). If it is not possible to remove all of the visible test article, the observation will be recorded in the study workbook.

The tissues will be placed into the incubator at standard culture conditions for an initial post-exposure incubation of 24±1 hours.

After the initial 24±1 hours post-treatment expression incubation, the tissues will be transferred into new wells of the 6-well plates filled with 0.9 mL fresh pre-warmed (37±1°C) Maintenance Medium and placed back into the incubator for the remainder of the 42 ± 2 hours post-treatment incubation (*i.e.*, 18±1 hours).

7.10 MTT Assay

MTT Preparation: A 10X stock of MTT prepared in PBS (filtered at time of batch preparation) will be thawed and diluted in warm MTT Addition Medium to produce the 1.0 mg/mL solution no more than two hours before use. Three hundred microliters (300 µL) of the MTT solution will be added to each designated well of a labeled 24-well plate.

After the total 42 ± 2 hours post-exposure expression incubation, each tissue will be blotted on a absorbent paper and transferred to the appropriate well containing 0.3 mL of MTT solution. The 24-well MTT plates will be incubated at standard culture conditions for 3 ± 0.1 hours.

After the 3 ± 0.1 hour incubation, the EpiDerm™ tissues will be submerged, gently swirled, and rinse media dumped in a beaker containing approximately 150 mL of CMF-DPBS. This process will be repeated for a total of 3 rinses. The tissues will then be blotted on absorbent paper, cleared of excess liquid, and transferred to a labeled 24-well plate containing 2.0 mL of isopropanol in each designated well. The plates will be covered with parafilm, and will be shaken for 2 - 3 hours at room temperature to extract the MTT. If necessary, the plates may be stored in the refrigerator (2-8°C) overnight (or up to 20 hours after the last tissue is harvested) prior to extracting the MTT. At the end of the extraction period, the cell culture insert will be pierced with forceps and gently agitated up and down in its extractant well. The tissue will then be lifted to allow the

extracted solution to flow back into the well from which the cell culture insert was removed, and the cell culture insert will be discarded. The extract solution will be mixed (homogenized by pipetting up and down 3 times) and **two x 200 µL aliquots** will be transferred to the appropriate wells of a 96-well plate. **Six x 200 µL** of isopropanol will be added to the wells designated as blanks. The absorbance at 570 nm (OD_{570}) of each well will be measured with a Molecular Devices Vmax plate reader with the AUTOMIX function selected.

7.11 Killed Controls for Assessment of Residual Test Article Reduction of MTT

In cases where the test article is shown to reduce MTT, test article that remains bound to the tissue after rinsing could result in a false MTT reduction signal. To demonstrate whether possible residual test article is acting to directly reduce the MTT, a functional check is performed using freeze-killed tissue to determine whether the test material is binding to the tissue and leading to a false MTT reduction signal.

Freeze-killed tissues will be prepared by placing untreated EpiDerm™ tissues in the -20±5°C freezer at least overnight, thawing to room temperature, and then refreezing. Once refrozen, the tissue may be stored indefinitely in the freezer. Freeze-killed tissues may be received prepared from MatTek Corporation. Once received at IIVS, the freeze-killed tissues will be warmed to room temperature, and then transferred to the -20±5°C freezer until use. To test for residual test article reduction, two killed tissues are treated with the test article in the same fashion as the viable tissues. All assay procedures will be performed in the same manner as for the viable tissue. Two negative control-treated killed controls will be tested in parallel since a small amount of MTT reduction is expected from the residual NADH and associated enzymes within the killed tissue, and will generate background reduction of MTT.

In some cases, the amount of MTT reduction in the treated killed control may be significant and the test article may be considered untestable in this system. The OD_{570} values from the killed controls will be analyzed as described in **§7.14**.

7.12 Colorant Controls (CC) for Assessment of MTT Interference

In cases where a test article is shown to interfere with the MTT measurement (see §7.7.3 *Assessment of Colored or Staining Materials*), colorant controls (CC) will be tested. **Ideally, the colorant controls will be tested concurrently with the definitive assay; however, in some cases (*e.g.*, insufficient tissues) follow-up testing of the colorant controls on a separate testing date may be warranted.** To determine whether the test article is interfering with the MTT measurement, at least one live colorant control (CC) tissue will be tested per test article. The CC tissues will be treated in the same manner as the live tissues with the exception that the tissues will incubated in 0.3 mL of Maintenance Medium instead of MTT for 3 ± 0.1 hours at standard culture conditions. After the 3 ± 0.1 hours incubation period, the tissues will be processed as outlined in section 7.10.

The CCs will be analyzed as described in §7.14.

7.13 Colorant Control-Killed Control (CC-KC)

In cases where a test article is shown to reduce MTT **and** also interfere with the MTT measurement, colorant control-killed controls (CC-KC) will be tested concurrently. The CC-KC will be tested to avoid a possible double correction for color interference. Two killed control tissues per test article and per exposure time will be used. The CC-KC tissues will be treated in the same way as the CC tissues (i.e. incubation in 0.3 mL of Maintenance Medium instead of MTT for 3 ± 0.1 hours at standard culture conditions). After the 3 ± 0.1 hours incubation period, the tissues will be processed as outlined in section 7.10.
The CC-KC will be analyzed as described in §7.14.

7.14 Data Analysis

MTT Analysis for SIT

All calculations will be performed using an Excel spreadsheet. The mean OD_{570} value of the blank wells will be calculated. Individual blank-corrected OD_{570} values for each of the duplicate aliquots of each test article or control tissue will be determined by subtracting the mean OD_{570} value of the blank wells from their individual OD_{570} values. The mean corrected OD_{570} value will be calculated for each individual test article or control tissue from the duplicate aliquots.

Corrected Individual Tissue OD_{570} = Individual Tissue OD_{570} – mean Blank OD_{570}

When applicable, the final corrected OD_{570} of the test article(s) will be determined using OD_{570} values for the killed controls (KC), colorant controls (CC), and/or colorant control-killed controls (CC-KC). The final corrected OD_{570} determination will be used to determine relative viability. *Note: any negative value that may be obtained for KC, CC or CC-KC will be equaled to zero for calculation purposes.*

If killed controls (KC) are used, the following additional calculations will be performed to correct for the amount of MTT reduced directly by test article residues. The mean raw OD_{570} value for the negative control killed control will be subtracted from the mean raw OD_{570} values for the test article-treated killed controls to determine a net OD_{570} value of the test article-treated killed controls.

Net OD_{570} for each test article KC = Mean Raw OD_{570} test article KC – Mean Raw OD_{570} negative control KC

The net OD_{570} values represent the amount of reduced MTT due to direct reduction by test article residues. The net amount of MTT reduction will be subtracted from the corrected OD_{570} values of the viable treated tissues to obtain a final corrected OD_{570} value. Although the algorithms discussed are performed to calculate the final endpoint analysis at the treatment group level, the same calculations can be applied to the individual replicates.

Corrected OD_{570} of the test article= Corrected test article OD_{570} (viable) – Net KC OD_{570}

If colorant controls (CC) are used, the OD_{570} value of the CC will be subtracted from the corrected OD_{570} values of the viable treated tissues to obtain a final corrected OD_{570} value.

Corrected OD_{570} of the test article= Corrected test article OD_{570} (viable) – CC OD_{570}

If colorant control-killed controls (CC-KC) are used, the OD_{570} value of the CC-KC will be added to corrected OD_{570} values of the viable treated tissues to obtain a final corrected OD_{570} value.

Corrected OD_{570} of the test article= Corrected test article OD_{570} (viable) + CC-KC OD_{570}

The corrected OD_{570} value(s) of the test article(s) for the KC, CC, and/or CC-KC will be used to determine the final corrected OD_{570} value.

Final Corrected OD_{570} of the test article=
Corrected test article OD_{570} (viable) – Net KC OD_{570} - CC OD_{570} + CC-KC OD_{570}

The final corrected OD_{570} values will then be used to determine the % of Control viabilities for each test article. The following % of Control calculations will be made for each individual tissue:

$$\% \text{ Viability} = \frac{\text{Mean Final Corrected } OD_{570} \text{ of Test Article or Control Tissue}}{\text{Corrected Mean } OD_{570} \text{ of Negative Control}} \times 100$$

The individual % of Control viability values will be tabulated for each individual tissue. Mean (and standard deviation) viability values will be calculated for each test article and control. Finally, the mean viability values will be plotted on bar graphs (with 1 standard deviation error bars) for each test article.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The assay will be accepted if the following criteria are met:

1) if the positive control (5% SDS) results in a mean tissue viability ≤ 20%;

2) if the mean OD_{570} value of the negative control tissues is ≥ 0.8 and < 2.8; and

3) if the standard deviation (SD) calculated from the individual % tissue viabilities of the 3 identically treated replicates of the negative or positive control are < 18%.

9.0 EVALUATION OF TEST RESULTS

The following Prediction Model has been endorsed by the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) for the prediction of skin irritation. A test article is predicted to be an irritant (GHS Category 1 or 2) if the mean relative viability of the three treated tissues is less than or equal to 50% of the mean viability of the negative control. *Additional testing (e.g., reconstructed human epidermis (RhE) OECD TG 431) in a tiered testing approach would be warranted to discriminate between GHS Category 1 and GHS Category 2.*

In Vitro Result	*In Vivo* Prediction	GHS Category
mean tissue viability ≤ 50%	Irritant (I)	Category 1 or 2*
mean tissue viability > 50%	Non-irritant (NI)	No Category

**- to discriminate between GHS Category 1 and 2, additional testing required*

Some test articles may produce a high standard deviation (SD), which may be due in part to the nature of the test article. Ideally, variability (SD) should be < 18%. Further, the individual tissue viabilities of the triplicate tissues should result in the same prediction (Irritant or Non-Irritant). If the SD of the three tissues is > 18% and/or the three tissues do not result in the same prediction, the results may be considered equivocal and further testing may be requested at the direction of the Study Director and in consultation with the Sponsor. In cases of borderline results (i.e. mean tissue viability of 50±5%), further testing may also be considered at the direction of the Study Director and in consultation with the Sponsor.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. A summary will be prepared reporting the mean viability values, and the irritation prediction for each test article. The report will also include a discussion of results. A copy of the protocol used for the study and any significant deviation(s) from the protocol will appear as a part of the final report.

11.0 RECORDS AND ARCHIVES

A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data, reports and specimens will be retained in the archives for a period of either a) 5 years, b) the length of time specified in the contract terms and conditions, or c) as long as the quality of the preparation affords evaluation, whichever is applicable.

12.0 TEST MATERIAL RETENTION

Unless indicated otherwise, all test articles provided by the sponsor and dose solutions used for testing or analysis before or during the course of the assay will be retained for one year after completion of the final report. These test articles and dose solutions may be disposed after this 1 year retention period according to IIVS' SOP.

13.0 PROTOCOL AMENDMENTS

When it becomes necessary to change the approved protocol for a specific study, the change and the reason for it should be put in writing and signed by the Study Director as soon as practical. When the change may impact the study design and/or execution, verbal agreement to make this change should be made between the Study Director and Sponsor. This document is then provided to the Sponsor and is attached to the protocol as an amendment.

14.0 REFERENCES

MatTek Corporation. Protocol for: IN VITRO EpiDerm™ SKIN IRRITATION TEST (EPI-200-SIT) for use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm (EPI-200-SIT). SOP MK-24-007-0023, Rev. 03/26/2012.
OECD Guidelines for The Testing of Chemicals. *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method (OECD TG 439), adopted 28 July 2015.

Shopsis, C. and Eng, B. *In vitro* ocular irritancy prediction: assays in serum-free medium correlate better with *in vivo* data. In Alternative Methods in Toxicology, Vol 6, A.M. Goldberg (Ed.), Mary Ann Liebert, Inc. NY, 253 (1988).

Berridge, M.V., Tan, A.S., McCoy, K.D., Wang, R. (1996) The Biochemical and Cellular Basis of Cell Proliferation Assays That Use Tetrazolium Salts. Biochemica 4:14-19.

15.0 APPROVAL

See Sponsor's email of 11 May 2018 for approval
SPONSOR REPRESENTATIVE DATE

(Print or Type Name)

IIVS STUDY DIRECTOR DATE 17 May 2018

PROTOCOL ATTACHMENT 1

IIVS Test Article Designation	Sponsor Designation
18AD37	

Test Article Handling and/or Preparation:

The test article will be tested neat. The test article was provided in a liquid nitrogen pressurized cylinder. Before being used in the step(s) of the assay, an aliquot of the test article needed for the assay will be removed from the cylinder, placed into a glass vial labeled according to the SOP(s) of the Testing Facility, and stored in the same conditions (15°C - 30°C) as the original container received from the Sponsor. After use, the remaining aliquoted sample will be discarded according to the disposal instruction in the Safety Data Sheet (SDS) provided by the Sponsor. The exact amount of the aliquoted test article and any specific aspects of the handling procedures will be captured in the study workbook and report.

The tissues treated with the test article will be cultured on plate(s) separated from the assay controls to avoid any contamination through possible evaporation that may occur.

REGULATORY REQUIREMENTS:

Will this study be conducted according to **GLPs**? [**X**] **YES** or [ ] **NO**

If **YES**, please indicate which agency(ies) guidelines are to be followed:
[ ] FDA; [**X**] EPA TSCA; [ ] EPA FIFRA; [ ] OECD; [ ] Other

APPENDIX B

EPIDERM ™ SIT COLORANT CONTROLS ASSESSMENT

Study No. 18AD37.050082
Plate: 1
Read Date: 05/2 /18 Read By: EW
Raw Data: Optical Density (OD_{570})

	1	2	3	4	5	6	7	8	9	10	11	12
A:												
B:												
C:	0.036	0.035										
D:												
E:												
F:												
G:												0.050
H:												0.065

Plate Map:

	1	2	3	4	5	6	7	8	9	10	11	12
A:												
B:												
C:	T3C1	T3C1										
D:												
E:												
F:												
G:												BLNK
H:												BLNK

Average Blank OD: 0.058

	Trial	Map Prefix	Average OD_{570} Values	Corrected OD_{570} (Avg. TA- Avg. Blank OD)	Possible MTT Interference? (Corrected OD570>0.08)
Test Article 18AD37	B1	T3C1	0.036	-0.022	No

Negative control (NC)	Negative Control (NC)
Positive control (PC)	Positive Control (PC)
Test Chemical	18AD37

Dosing Date:	23-May-18

96 WELL PLATE MAP

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12	
A	**BLANK**	**BLANK**	**BLANK**	**BLANK**	**BLANK**	**BLANK**							
B	**NC**	**PC**			**18AD37**								Tissue1
C	**NC**	**PC**			**18AD37**								
D	**NC**	**PC**			**18AD37**								Tissue2
E	**NC**	**PC**			**18AD37**								
F	**NC**	**PC**			**18AD37**								Tissue3
G	**NC**	**PC**			**18AD37**								
H													

RAW DATA

PLATE 1

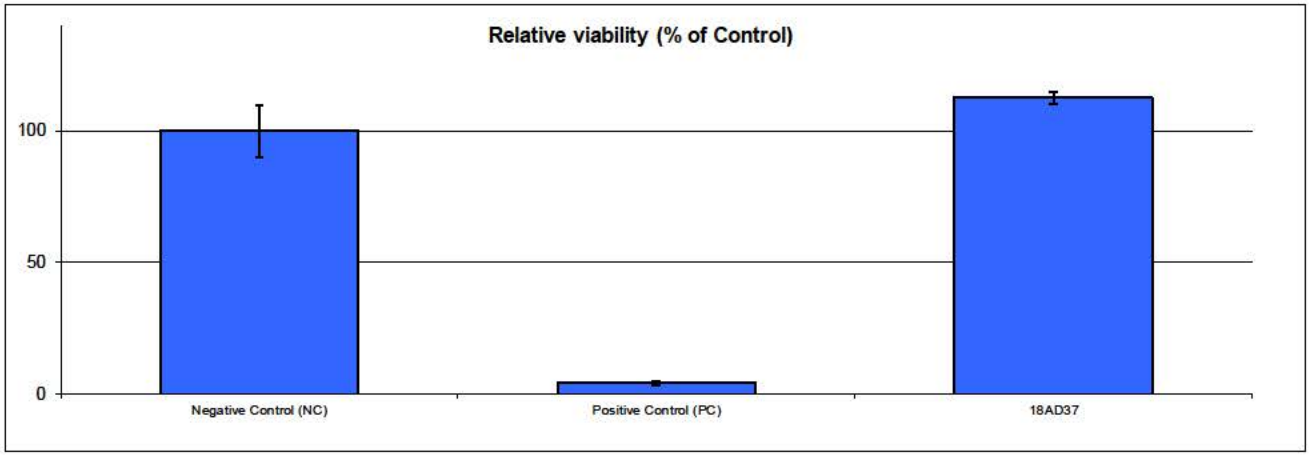
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.035	0.033	0.034	0 034	0.036	0.035							
B	1.412	0.081			1.624								Tissue1
C	1.423	0.083			1.605								
D	1.262	0.085			1.556								Tissue2
E	1.299	0.085			1.555								
F	1.537	0.107			1.612								Tissue3
G	1.578	0.108			1.604								
H													

COMMENTS

RESULTS

Dosing Date:	23-May-18

Blank	0.035
	0.033
	0.034
	0.034
	0.036
	0.035
Mean	0.035



Chemical Code	Tissue n	Raw OD Aliq. 1	Raw OD Aliq. 2	Blank corrected OD data Aliq. 1	Blank corrected OD data Aliq. 2	mean of OD (aliquots)	% of viability
Negative Control (NC)	1	1.412	1.423	1.378	1.389	1.383	99.9
	2	1.262	1.299	1.228	1.265	1.246	90.0
	3	1.537	1.578	1.503	1.544	1.523	110.0
Positive Control (PC)	1	0.081	0.083	0.047	0.049	0.048	3.43
	2	0.085	0.085	0.051	0.051	0.051	3.65
	3	0.107	0.108	0.073	0.074	0.073	5.27
18AD37	1	1.624	1.605	1.590	1.571	1.580	114.2
	2	1.556	1.555	1.522	1.521	1.521	109.9
	3	1.612	1.604	1.578	1.570	1.574	113.7

	mean of OD	SD of OD	mean of viabili ies [%	SD of viabili ies	CV % [%]
Negative Control (NC)	1 384	0.139	100.0	10 01	10 01
Positive Control (PC)	0 057	0.014	4.12	1.01	24.45
18AD37	1 558	0.032	112.6	2.34	2.08

APPENDIX C

gibco

Certificate of Analysis

Dulbecco's Phosphate Buffered Saline (1X), liquid
– Calcium Chloride
– Magnesium Chloride

Catalog Number:	14190
Lot Number:	1930100
Storage Temperature:	15 to 30C
Expiration Date:	2021-01

For in vitro diagnostic use.

Sterile filtered (0.1 um)

TEST	SPECIFICATION	RESULT	UNITS
[1] Endotoxin Testing	Tested	<0.03	EU/mL
[2] Osmolality	>=270 to <=300	282	mOsm/kg
[3] pH	>=7.0 to <=7.3	7.2	
[4] Sterility Testing	Negative	Negative	

Read SDS

GIBCO brand, Thermo Fisher Scientific cell culture liquid products are prepared by an aseptic process for which each step has been validated to ensure that all products meet the industry standard sterility assurance level of 10^-3; i.e. product that demonstrates a contamination level of no more than 1 of 1,000 units during the manufacturing process. The highest level of sterility assurance (equal to or greater than 10^-6) cannot be achieved without terminal sterilization which is harmful to the performance of cell culture products.

Barbara J Schueler

Quality Systems Department Date: 29-Jan-2018

REFERENCES:

1 Current United States Pharmacopeia, <85> Bacterial Endotoxins Test.
2 Thermo Fisher Scientific Specifications.
3 Thermo Fisher Scientific Specifications.
4 Current edition of USP.

Life Technologies Corporation
3175 Staley Rd., Grand Island, New York 14072, United States

SIGMA-ALDRICH®

sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Sodium dodecyl sulfate - ReagentPlus®, ≥98.5% (GC)

Product Number:	**L4509**
Batch Number:	**SLBS5305**
Brand:	SIAL
CAS Number:	151-21-3
MDL Number:	MFCD00036175
Formula:	C12H25NaO4S
Formula Weight:	288.38 g/mol
Quality Release Date:	13 DEC 2016
Recommended Retest Date:	DEC 2019

Test	Specification	Result
Appearance (Color)	White to Off White	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless to Very Faint Yellow	Colorless
Solubility (Turbidity) 200 mg/mL, H2O	Clear	Clear
Water (by Karl Fischer)	≤ 2.0 %	0.9 %
Carbon	49.2 - 50.7 %	49.5 %
Sodium (Na)	7.0 - 9.0 %	7.6 %
Purity (GC)	≥ 98.5 %	99.3 %
Purity by Titration	≥ 98.5 %	99.8 %

Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri US

C4

FINAL REPORT

Study Title

IN VITRO SKIN CORROSION ASSAY USING THE EPIDERM™ SKIN MODEL (EPI-200): 3 - AND 60 - MINUTE EXPOSURE PROTOCOL

Test Articles

Authors

Gertrude-Emilia Costin, Ph.D., M.B.A.
Elizabeth Willier, M.S.

Study Completion Date

28 June 2018

Performing Laboratory

Institute for In Vitro Sciences, Inc.
30 W. Watkins Mill Road, Suite 100
Gaithersburg, MD 20878

Study Number

18AD37.050054

Laboratory Project Number

9779

Sponsor Study Number

TABLE OF CONTENTS

STATEMENT OF COMPLIANCE

The *In Vitro* Skin Corrosion Assay Using The EpiDerm™ Skin Model (EPI-200): 3 - And 60 - Minute Exposure Protocol of the test article,

was conducted in compliance with the U.S. EPA GLP Standards 40 CFR 792, in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the assay controls have not been determined by the testing facility. However, certificates of analysis from suppliers for the assay controls are included in Appendix C.

The stability of the test article and of the assay controls under the storage conditions at the testing facility and under the actual test conditions has not been determined by the testing facility and is not included in the final report.

Gertrude-Emilia Costin, Ph.D., M.B.A.
Study Director

28 June 2018
Date

QUALITY ASSURANCE STATEMENT

Study Title: *In Vitro* Skin Corrosion Assay Using the EpiDerm™ Skin Model (EPI-200): 3- and 60- Minute Exposure Protocol

Study Number: 18AD37.050054

Study Director: Gertrude-Emilia Costin, Ph.D., M.B.A.

A random sampling approach was used to select at least one in-process, laboratory phase to inspect for this study. The Quality Assurance Unit inspections specific to this study are listed in the table below. Procedures, documentation, equipment records, etc., were examined in order to assure that the study was performed in accordance with the U.S. EPA GLP Standards 40 CFR 792 and to assure that the study was conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director and Management
Protocol and Initial Paperwork	23-Apr-18	23-Apr-18
Treatment of tissues with test article and positive control (3 minute exposure)	03-May-18	03-May-18
Final Report, Data and Protocol Amendment I	27-Jun-18 28-Jun-18	28-Jun-18

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Megan Conahan 28 Jun 18

Megan Conahan, B.S.
Quality Assurance

Date

SIGNATURE PAGE

***IN VITRO* SKIN CORROSION ASSAY USING THE EPIDERM™ SKIN MODEL (EPI-200): 3 - AND 60 - MINUTE EXPOSURE PROTOCOL**

Initiation Date:	20 April 2018
Completion Date:	28 June 2018
Sponsor:	
Sponsor's Representative:	
Testing Facility:	Institute for In Vitro Sciences, Inc. 30 W. Watkins Mill Road, Suite 100 Gaithersburg, MD 20878
Archive Location:	Institute for In Vitro Sciences, Inc. Gaithersburg, MD 20878
Study Director:	28 June 2018 Gertrude-Emilia Costin, Ph.D., M.B.A. Date
Director of Laboratory Operations:	Greg Mun, B.A.

TEST ARTICLE RECEIPT

IIVS Test Article Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions*
18AD37		clear colorless non-viscous liquid	6 April 2018	room temperature

* - Protected from exposure to light

IN VITRO SKIN CORROSION ASSAY USING THE EPIDERM™ SKIN MODEL (EPI-200): 3 - AND 60 - MINUTE EXPOSURE PROTOCOL

INTRODUCTION

The purpose of this study was to assess the potential skin corrosivity of the test article, supplied by in the EpiDerm™ Kit (MatTek Corporation). The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) conversion assay, which measures the NAD(P)H-dependent microsomal enzyme reduction of MTT (and to a lesser extent, the succinate dehydrogenase reduction of MTT) to a blue formazan precipitate, was used to assess cellular metabolism after test article exposure[1]. The protocol is consistent with the OECD Test Guideline 431 "*In Vitro* Skin Corrosion: Human Skin Model Test"[2].

The method utilizes a 3-minute exposure for a corrosive classification and a 60-minute confirmatory exposure for materials found to be non-corrosive at the 3-minute exposure. Viable cells reduce the yellow, soluble, oxidized form of the MTT to the blue-black, insoluble, reduced form. The reduced dye is extracted from the tissue with isopropanol, and the amount of reduced dye is determined spectrophotometrically. The relative viability of the treated tissues is calculated as a percentage of the negative control viability from the absorbance data by dividing the corrected test article-treated tissue absorbance by the corrected control tissue absorbance, and multiplying by 100. Test materials which reduce tissue viability to <50% within 3 minutes are considered corrosive by this method. In addition, test materials which result in tissue viability of ≥50% after a 3-minute exposure, but result in tissue viability of <15% after a 60-minute exposure are also classified corrosive. Test materials which result in tissue viabilities of ≥50% after a 3-minute exposure and ≥15% after a 60-minute exposure are classified non-corrosive. Furthermore, sub-classification of corrosive materials is possible using the 3 minute exposure time as follows: a sub-category classification of 1A is assigned if the viability is <25%, and 1B/1C if the viability is ≥ 25%.

The laboratory phase of the study was conducted from 3 May 2018 to 4 May 2018 at the Institute for In Vitro Sciences, Inc. The test article was tested in a definitive assay to determine the relative viability of the tissues exposed to the test article compared to the tissues exposed to the negative control.

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1 Berridge, M.V., Tan, A.S., McCoy, K.D., Wang, R. (1996) The Biochemical and Cellular Basis of Cell Proliferation Assays That Use Tetrazolium Salts. **Biochemica** 4:14-19.

2 OECD Test Guideline 431 "In Vitro Skin Corrosion: reconstructed human epidermis (RHE) test method", Adopted 29 July 2016.

MATERIALS AND METHODS

Receipt of the EpiDerm™ Skin Model

Upon receipt of the EpiDerm™ Skin Bioassay Kit, the solutions were stored as indicated by the manufacturer. The EpiDerm™ tissues were stored at 2-8ºC until used. On the day of dosing, an appropriate volume of EpiDerm™ assay medium was removed and warmed to approximately 37ºC. Nine-tenths (0.9) mL of assay medium were aliquotted into the wells of each 6-well plate. The six-well plates were labeled to indicate test article and exposure time. The EpiDerm™ tissues were inspected for air bubbles between the agarose gel and cell culture insert prior to opening the sealed package. Tissues with air bubbles covering greater than 50% of the cell culture insert area were not used. The 24-well shipping containers were removed from the plastic bag and their surfaces were disinfected with 70% ethanol. The EpiDerm™ tissues were transferred aseptically into the 6-well plates. The EpiDerm™ tissues were then incubated in the dark at 37±1ºC in a humidified atmosphere of 5±1% CO_2 in air (standard culture conditions) for at least one hour. The medium was then aspirated and 0.9 mL of fresh medium were added to each assay well below the EpiDerm™ tissues. The plates were returned to the incubator until treatment was initiated. Upon opening the bag, any remaining unused tissues were briefly gassed with an atmosphere of 5% CO_2/95% air and placed back at 2-8ºC for later use.

Preparation and Delivery of Test Article

The test article was handled in a fully vented hood. The test article was tested neat. The test article was provided in a liquid nitrogen pressurized cylinder. An aliquot needed for the assay (~1.0 mL) was removed from the cylinder and placed into a glass vial, which was stored at room temperature. The tissues treated with the test article were cultured on separate plates from the assay controls. Fifty (50) µL of the test article were applied directly on the tissue so as to cover the upper surface (epithelial side). The tissues designated to the negative control were treated with 50 µL of sterile, deionized water. The tissues designated to the positive control, 8N KOH (Sigma), were tested using the same method. All exposure conditions were documented in the study workbook.

Assessment of Direct Test Article Reduction of MTT

The test article was added to a 1.0 mg/mL MTT (Sigma) solution in warm Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine (MTT Addition Medium) to assess its ability to directly reduce MTT. Approximately 50 µL of the test article were added to 1 mL of the MTT solution, and the mixture was incubated at standard culture conditions for at least one hour. A negative control, 50 µL of sterile, deionized water (Quality Biological), was tested concurrently. If the MTT solution color turned blue/purple, the test article was presumed to have reduced the MTT.

In cases where the test article was shown to reduce MTT, only those test articles that remained bound to the tissue after rinsing, resulting in a false MTT reduction signal, could present a problem. To evaluate whether residual test article was binding to the tissue and leading to a false MTT reduction signal, a functional check (using freeze-killed control tissue) was performed as described in the section labeled "Killed Controls (KC)".

The test article, was not observed to directly reduce MTT in the absence of viable cells.

The positive control, 8N potassium hydroxide (8N KOH), is known to directly reduce MTT in the absence of viable cells. Therefore, a killed control experiment was performed concurrently in the definitive assay to determine the extent of the direct MTT reduction (if any) by the positive control in non-viable freeze-killed tissues.

Assessment of Colored or Staining Materials

Approximately 50 µL were added to 2.0 mL isopropanol in 6-well plates and placed on an orbital place shaker for 2-3 hours at room temperature. After shaking, 200 µL aliquots of the isopropanol solutions and two blank samples of isopropanol were transferred to a 96-well plate and the absorbance was measured with a plate reader at the MTT measurement wavelength (550 nm).

The absorbance of the test article samples was determined by subtracting the mean isopropanol blank value from the absorbance of the test article samples. If the OD_{550} of the test article sample is > 0.08, the material has to be considered as possibly interacting with the MTT measurement.

The test article, was not considered to have probable photometric MTT interference.

pH Determination

The pH of the test article was measured using pH paper (EMD Millipore Corporation). Initially, the test article was added to pH paper with a 0-14 pH range in 1.0 pH unit increments to approximate a narrow pH range. Next, the test article was added to pH paper with a narrower range of 0-6 pH units with 0.5 pH unit increments, to obtain a more accurate pH value. The pH value obtained from the narrower range pH paper is presented in Table 1.

Skin Corrosion Assay

The test and control articles were tested by treating four EpiDerm™ tissues per material. Two tissues were used to assess viability after the 3-minute exposure, and two were used to assess viability after the 60-minute exposure. Fifty (50) microliters of the test article were applied topically on the EpiDerm™ tissue. Fifty (50) microliters of the negative and positive controls were applied topically on the EpiDerm™ tissue. The three-minute exposure time began as soon as the material was spread onto the tissue. This short exposure time precluded treating more than a small number of tissues at once. The cultures exposed for 3 minutes were held at room temperature during dosing, while the cultures exposed for the 60 minutes were incubated at standard culture conditions until the completion of the exposure time.

A 1.0 mg/mL solution of MTT in warm MTT Addition Medium was prepared no more than 2 hours before use. Three hundred (300) µL of MTT reagent solution were added to designated wells in a pre-labeled 24-well plate. The plate was held in the incubator until tissues were added. After the appropriate exposure time, the EpiDerm™ tissues were extensively rinsed with warm (approximately 37ºC) Calcium and Magnesium-Free Dulbecco's Phosphate Buffered Saline ($Ca^{++}Mg^{++}$-Free DPBS) and the wash medium was decanted. The EpiDerm™ tissues were transferred to the appropriate wells after rinsing. The plates were incubated at standard culture conditions for 3 ± 0.1 hours.

After the incubation period with MTT solution, the EpiDerm™ tissues were blotted on absorbent paper, cleared of excess liquid, and transferred to a pre-labeled 24-well plate containing 2.0 mL of isopropanol in each designated well. The plates were covered with paraffin film and stored in the refrigerator (2-8ºC) until the last exposure time was harvested. Then the plates were shaken for 2 - 3 hours at room temperature.

At the end of the extraction period, the liquid within the cell culture inserts was decanted into the well from which the cell culture insert was taken. The extract solution was mixed and 200 µL were transferred to the appropriate wells of a 96-well plate. Two hundred (200) µL of isopropanol were placed in the two wells designated as the blanks. The absorbance at 550 nm (OD_{550}) of each well was measured with a Molecular Devices Vmax plate reader.

Killed Controls (KC)

To evaluate whether residual test article was binding to the tissue and leading to a false MTT reduction signal, a functional check (using freeze-killed control tissue) was performed. Freeze-killed tissues were received from MatTek Corporation, and were stored in the freezer until use.

For the positive control, 8N KOH, duplicate killed tissues were treated in the normal fashion for 3 and 60 minutes. The rinsing, MTT exposure, and solvent extraction procedures were performed exactly as described for the viable tissues. Duplicate killed-control tissues were treated with the negative control for 3 and 60 minutes. A small amount of MTT reduction is expected from the residual NADH and associated enzymes within the killed tissue. This background reduction of MTT will be compared to the MTT reduction observed in the test article-treated killed-control tissues using calculations described in the Presentation of Data.

Presentation of Data

The raw absorbance values were captured. The mean OD_{550} value of the blank wells was calculated. The corrected mean OD_{550} values of the negative control exposure time were determined by subtracting the mean OD_{550} value of the blank wells from the mean OD_{550} values of each negative control exposure time. The average corrected mean OD_{550} values of the 3 and 60- minute negative controls was calculated for use in the subsequent % of Control calculations. The corrected OD_{550} values of the individual test article and positive control-treated tissues at both exposure times were determined by subtracting the mean OD_{550} value of the blank wells from each of their individual OD_{550} values. All calculations were performed using an Excel spreadsheet.

Since killed controls (KC) were used, the following additional calculations were performed to correct for the amount of MTT reduced directly by the positive control residues. The raw OD_{550} value for the negative control killed control was subtracted from the raw OD_{550} values for the positive control-treated killed controls (at both exposure times), to determine the net OD_{550} values.

Net OD_{550} test article or positive control = raw OD_{550} test article or positive control KC – raw OD_{550} negative control KC

The net OD_{550} values represent the amount of reduced MTT due to direct reduction by positive control residues. The net OD_{550} values were subtracted from the corrected mean OD_{550} values of the viable test article-treated EpiDerm™ tissues, to obtain a final corrected OD_{550}

value.

$$\text{Final corrected } OD_{550} = \text{corrected positive control } OD_{550} \text{ (viable)} - \text{net } OD_{550} \text{ positive control (KC)}$$

The individual viability values were calculated as follows:

$$\% \text{ Viability} = \frac{\text{Final corrected } OD_{550} \text{ of Test Substance or Positive Control Exposure time}}{\text{Average corrected mean } OD_{550} \text{ of the 3-minute and 60-minute of Negative Controls}} \times 100$$

The standard deviation (SD) of the tissue viability was then be calculated. The following % Viability (% of Control) calculations were made:

$$\% \text{ Viability} = \frac{\text{Final corrected mean } OD_{550} \text{ of Test Substance or Positive Control Exposure time}}{\text{Average corrected mean } OD_{550} \text{ of the 3-minute and 60-minute of Negative Controls}} \times 100$$

Finally, the upper limit and lower limit range of tissue viability were calculated as follows:

Upper limit = % Viability (mean) of test substance or positive control exposure time + (2 x SD)
Lower limit = % Viability (mean) of test substance or positive control exposure time – (2 x SD)

Evaluation of Test Results

If the test substance reduced tissue viability to <50% of the negative control value after a 3-minute exposure, the test substance was considered corrosive. In addition, test materials which resulted in tissue viability ≥ 50% after a 3-minute exposure but <15% after a 60-minute exposure were also classified as corrosive. Test materials which resulted in tissue viability ≥ 50% after 3-minute exposure and ≥ 15% after 60-minute exposure would be classified as non-corrosive. Furthermore, sub-classification of corrosive materials is possible using the 3 minute exposure time as follows: a sub-category classification of 1A is assigned if the viability is <25%, and 1B/1C if the viability is ≥ 25%.

Criteria for Determination of a Valid Test

The assay was accepted if: the positive control resulted in a corrosive classification (i.e., <50% cell viability compared to negative controls, after a 3-minute exposure and/or <15% cell viability compared to negative controls after a 60-minute exposure); and if the mean OD_{550} value of the negative control tissues was ≥ 0.8 and < 2.8.

RESULTS AND DISCUSSION

MTT Assay

The test article, was tested in the EpiDerm™ Corrosivity Assay. Two tissues were used to assess viability after a 3-minute exposure, and two tissues were used to assess viability after a 60-minute exposure. Negative and positive controls were tested in parallel. Table 1 summarizes the mean % viability results for the test articles and the positive control. The raw and analyzed data are presented in Appendix B. The classification of the positive control, 8N KOH, was determined to be corrosive thereby meeting the acceptance criterion.

The test article, was not observed to directly reduce MTT in the absence of viable cells. The positive control, 8N potassium hydroxide (8N KOH), is known to directly reduce MTT in the absence of viable cells. Therefore, a killed-control experiment was performed. The results of the killed control experiment showed that there was significant direct MTT reduction in the positive control-treated killed controls. Additional calculations were performed to correct for the amount of MTT reduced directly by the positive control and test article residues as described in the Presentation of Data section.

The test article, was not determined to be a colorant (was not considered to have potential interference with the MTT measurement).

Table 1

IIVS Test Article Number	Sponsor's Designation	Conc.	Exposure Time	% Viability	*Corrosive?	Sub-Category^	pH
18AD37		Neat	3 minutes	104.7	Non-Corrosive	NA	5.0
			60 minutes	105.1			
Positive Control	KOH	8N	3 minutes	12.0	Corrosive	1A	NA
			60 minutes	13.0			

NA – Not Applicable DpH – Test article discolored the pH paper

* - A material is considered corrosive if the percent viability is < 50% at the 3-minute exposure, or ≥ 50% at the 3-minute exposure and < 15% at the 60-minute exposure

^ - Using the 3 minute exposure time, a subcategory classification of 1A is assigned if the viability is <25%, and 1B/1C if the viability is ≥ 25%

Deviations:

1. On 2 May 2018, there was a power failure at the Testing Facility. The power was restored around 7:30 am. The temperatures of the Kenmore and Thermo Scientific Freezer were between +2 to -4 ºC at 7:42 am. This is a deviation from SOP0513.R7 that specifies acceptable freezer temperatures as -20±5ºC. This deviation affected storage conditions of any reagents stored at -20±5ºC. The temperature was in range (-16ºC) at 10:14 am. The temperatures of the Fridgidaire, VWR, and Thermo Scientific refrigerators were between 9 to 10ºC at 7:41 to 7:43 am. This power failure affected storage conditions of any tissues and reagents stored at 2-8ºC. The responses of the assay positive and negative control were in line with the historical ranges at IIVS. Therefore, the deviations are not considered to have

adversely affected the accuracy of the study.

2. The protocol states that to test for residual test article-mediated MTT reduction, two killed tissues will be treated with the positive control or test article concurrently with the viable tissues. During the study conduct, a significant difference in the MTT reduction on the second killed control tissue treated with the assay positive control for 3 minutes (raw OD_{550} value of 0.115 vs. 0.341 for the duplicate tissue). Therefore, another killed control experiment was conducted in the same day using duplicate tissues from the same lot. The raw OD_{550} values for the additional killed control tissues included in the study were 0.339 and 0.411, respectively, and comparable with the historical results. Under the circumstances, only the 3 OD_{550} corrected values for the 3 minutes killed control positive control that were comparable with historical values were averaged. The deviation is not considered to have an adverse impact on the study accuracy.

APPENDIX A

IN VITRO SKIN CORROSION ASSAY USING THE EPIDERM™ SKIN MODEL (EPI-200): 3 - AND 60 - MINUTE EXPOSURE PROTOCOL

1.0 PURPOSE

The purpose of this study is to evaluate the potential skin corrosivity of the test article by measuring the conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a formazan product by EpiDerm™ tissues after exposure to the test article(s). The method utilizes a 3-minute exposure for corrosive materials detection and a 60-minute confirmatory exposure for materials found to be non-corrosive by the 3-minute exposure. The protocol is consistent with the OECD Test Guideline 431 (TG 431) "*In vitro* skin corrosion: reconstructed human epidermis (RHE) test method". Furthermore, the OECD TG 431 allows the sub-categorization of corrosive substances and mixtures into optional Category 1A and Category 1B/1C in accordance with UN GHS.

2.0 SPONSOR

2.1 Name:

2.2 Address:

2.3 Representative:

2.4 Sponsor Study No.:

3.0 IDENTIFICATION OF TEST ARTICLE(S) AND ASSAY CONTROLS

3.1 Test Article(s): See Protocol Attachment 1

(Test substances should be shipped along with Material Safety Data Sheets and appropriate shipping hazard reporting forms, whenever available.)

3.2 Assay Controls: Positive: 8N KOH

Negative: sterile deionized water

3.3 Determination of Strength, Purity, etc.

3.3.1 For GLP studies, the Institute for In Vitro Sciences, Inc. (IIVS) will attempt to secure documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions from the Sponsor. If the Sponsor is unable to provide such information, IIVS will retain documentation supporting attempts to obtain this information with the study file and the final report will be generated with an exception noted in the Statement of Compliance.

3.3.2 IIVS will be responsible for the documentation of the analytical purity and composition of the negative and positive controls. This may be accomplished by maintaining a certificate of analysis from the supplier.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Institute for In Vitro Sciences, Inc.
4.2	Address:	30 W. Watkins Mill Road, Suite 100 Gaithersburg, MD 20878
4.3	Study Director:	Gertrude-Emilia Costin, Ph.D., M.B.A.

5.0 TEST SCHEDULE

5.1	Proposed Experimental Initiation Date:	30 April 2018
5.2	Proposed Experimental Completion Date:	18 May 2018
5.3	Proposed Report Date:	13 July 2018

6.0 TEST SYSTEM

The EpiDerm™ Model (EPI-200) (MatTek Corporation, Ashland, USA) that will be used in this study consists of normal, human-derived epidermal keratinocytes which have been cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered *stratum corneum* containing intercellular lamellar lipid layers arranged in patterns analogous to those found *in vivo*. The EpiDerm™ Model incorporates several features which make it advantageous in the study of potential dermal corrosivity. First, the test system uses a serum-free medium which eliminates the possibility of serum protein and test article interaction (Shopsis and Eng, 1988). Secondly, the target cells are epithelial, derived from human skin (Cannon *et al.*, 1994). Third, since the tissue has a functional *stratum corneum*, the test materials are applied directly to the tissue surface, at air interface, so that undiluted and/or end use dilutions can be tested directly (Harbell *et al.*, 1994). Prior to use, each 6, 24 and 96-well plate will be uniquely identified with a number written in permanent marker with the test article identification or control treatment group.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The *in vitro* skin corrosion assay is used to determine the potential skin corrosivity of test materials. The pH of the test article will be determined, if possible, using pH paper. The method consists of exposing the *stratum corneum* surface (epithelial side) of the tissue to the test material. After a 3- or 60-minute exposure (two tissues per exposure), the test article is removed from the tissue by rinsing with calcium and magnesium-free Dulbecco's phosphate buffered saline (CMF-DPBS). The rinsed tissue is then incubated for 3 hours in an MTT dye solution to determine the degree of cytotoxicity (cell death) caused by exposure to the test material. Viable cells reduce the yellow, soluble oxidized form of the

MTT to the blue-black insoluble form. The reduced dye is extracted from the tissue with isopropanol and the amount of reduced dye is determined spectrophotometrically. The relative viability of the treated tissues is calculated as a percentage of the negative control viability. Test articles that reduce tissue viability to <50%, after a 3-minute exposure, are classified corrosive by this method. In addition, test articles which result in tissue viability ≥50% after a 3-minute exposure **and** <15% after a 60-minute exposure are also classified corrosive. Test articles which result in tissue viabilities of ≥50% after a 3-minute exposure and ≥15% after a 60-minute exposure are classified non-corrosive (Liebsch *et al.*, 2000).

This assay is a modification of the procedures supplied by MatTek Corporation (SOP MK-24-007-0024).

This assay has been formally validated under the auspices of the European Centre for the Validation of Alternative Methods (Barratt *et al.*, 1998; Fentem *et al.*, 1998). This study conforms to the OECD Test Guideline 431, "*In vitro* skin corrosion: reconstructed human epidermis (RHE) test method", adopted 29 July 2016.

7.1 Media and Reagents

7.1.1 EpiDerm™ Assay Medium: Supplied by MatTek Corporation

7.1.2 EpiDerm™ Skin Model (EPI-200): Supplied by MatTek Corporation

7.1.3 Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine (MTT Addition Medium)

7.1.4 MTT (3-[4,5 - dimethylthiazol-2-yl] - 2,5 - diphenyltetrazolium bromide) Solution: 1.0 mg/mL MTT in MTT Addition Medium

7.1.5 Sterile Calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (CMF-DPBS)

7.1.6 Extraction Solvent: Isopropanol

7.1.7 Sharp spoon (for delivering approximately 25 mg of solids) Aesculap, Catalog No. FK 623R

7.2 Environmental Conditions

Throughout this protocol, ranges for test material and test system exposure or incubation conditions (e.g., temperature, humidity, CO_2,) are presented. These ranges describe the equipment performance specifications under static conditions (i.e., in the absence of frequent opening of equipment doors, accessing chambers, changing loads, etc.), as presented in the relevant equipment SOPs.

7.3 Preparation and Delivery of Test Article

Test articles will be handled in a fully vented hood. Test articles will generally be

tested neat. End use concentrations or other forms may be used as directed by the Sponsor. Liquid test articles will be delivered with a positive displacement pipette. Fifty microliters (50 µL) of pipettable substances, such as liquids, gels, creams, and foams, will be applied directly on the tissue so as to cover the upper surface. To aid in filling the pipet for pipettable materials that are viscous, the test article may first be transferred to a syringe. Extreme caution must be used in filling the pipet since the test articles may be corrosive. The pipet tip of a positive displacement pipet will be inserted into the dispensing tip of the syringe so that the material can be loaded into the displacement tip under pressure. Simultaneously, the syringe plunger is depressed as the pipet piston is drawn upwards. If air bubbles appear in the pipet tip, the test article should be removed (expelled) and the process repeated until the tip is filled without air bubbles. This method should be used for any test articles that cannot be easily drawn into the pipet such as gels and solid test articles which are creamed. A dosing device may be placed over the test article to assure even spreading, if required. Materials that are too viscous to spread over the tissue will first be spread onto the flat end of a dosing device. The dosing device will be put into the cell culture insert to bring the test article in contact with the tissue. When the dosing device is used, approximately 50 µL of the test article will be applied to the dosing device so as to cover the dosing surface. The sample should be spread to form a relatively smooth even layer on the surface of the dosing device to maximize uniform contact with the tissue. Certain solids can be pre-softened by creaming the sample in a weigh boat. The softened portion can be transferred to a syringe affixed with a three way stopcock attached to a second syringe. The sample is pushed from syringe to syringe until it is of a consistency that can be pipetted.

Dry solids or powders may be ground with a mortar and pestle to a fine powder if needed in order to improve the consistency. The solid test article will be placed directly onto the tissue at approximately 25 mg/tissue using a 25 mg sharp spoon (Aesculap, Cat. No. FK 623R). The sharp spoon will be filled with test article, and leveled by gently stroking away excess test article using a rod-shaped instrument. Care will be taken to avoid packing the material into the spoon. The contents of the spoon will be poured over the tissue surface. Each EpiDerm™ tissue treated with a solid test article will receive 25 µL of sterile, deionized water applied directly onto the test article. The test article will be gently mixed, and spread over the tissue surface using a sterile bulb-headed rod if needed.

The exact exposure conditions used for other test article forms will be determined after consultation with the Sponsor and/or the Study Director. All exposure conditions will be documented in the study workbook.

The stability of the test article under the storage conditions at the testing facility and under the actual experimental conditions will not be determined by Institute for In Vitro Sciences, Inc. (IIVS).

7.4 Route of Administration

Test articles will be administered by topical application to the tissue. When possible, liquids and pastes will be applied directly to the tissue surface. When the material is

too viscous to spread, it will first be applied to a dosing device (see section §7.3). In all cases, the test article will be placed onto the apical side (*stratum corneum*) of the tissue.

7.5 Controls

The assay will include a negative control and a positive control. The tissues designated for the negative control will be treated with 50 µL of sterile, deionized water. The tissues designated for the positive control will be treated with 50 µL of 8 Normal potassium hydroxide (8N KOH). The negative and positive controls will be exposed to the EpiDerm™ tissues at two exposure times of 3 – and 60 – minutes. **In addition, a killed control experiment will be performed (see Section 7.10) concurrently for the positive control at the 3 – and 60 – minute exposure times since KOH is a direct MTT reducer.**

7.6 pH Determination

The pH of the neat liquid test article (and/or dosing solution as appropriate) will be determined, if possible. The pH will be determined using pH paper (for example, with a pH range of 0 – 14 to estimate, and/or a pH range of 5 – 10 to determine a more accurate value). The typical pH increments on the narrower range pH paper are approximately 0.3 to 0.5 pH units. The maximum increment on the pH paper is 1.0 pH units.

7.7 Assessment of Direct Test Article Reduction of MTT

Prior to conducting any assays with viable tissues, the ability of each test article to directly reduce MTT will be assessed. A 1.0 mg/mL MTT solution will be prepared in warm MTT Addition Medium as described in §7.10. Approximately 50 µL (liquid test articles) or approximately 25 mg (solid test articles) will be added to 1 mL of the MTT solution and the mixture will be incubated in the dark at 37°C ± 1°C for one to three hours. A negative control, 50 µL of sterile, deionized water, will be tested concurrently. If the MTT solution color turns blue/purple relative to the negative control, the test article is presumed to have reduced the MTT and a killed control experiment will be performed as described in section §7.11. Water insoluble test materials may show direct reduction (darkening) only at the interface between the test article and the medium.

The MTT direct reduction test for the test article(s) may have been previously performed in an independent study. In such cases, the results of the MTT direct reduction test may be used for this specific study and the initial study will be referenced.

7.8 Assessment of Colored or Staining Materials

Prior to conducting any assays with viable tissues, the test article(s) ability to interfere with the photometric MTT measurement will be assessed. Each test article should be checked for its colorant properties (*e.g.,* their ability to absorb light

significantly at the wavelength used for the MTT determination).

Approximately 50 µL (liquid test articles) or one leveled spoonful (approximately 25 mg) (solid test articles) will be added to 2.0 mL isopropanol in 6-well plates and placed on an orbital plate shaker for 2-3 hours at room temperature. After shaking, 200 µL aliquots of the isopropanol solutions and two blank samples of isopropanol will be transferred to a 96-well plate and the absorbance will be measured with a plate reader at the MTT measurement wavelength (550 nm).

Some test articles may precipitate or become cloudy after addition to the isopropanol and interfere with the optical density (OD) measurement. In such cases, after the 2-3 hour shaking period, the test article-isopropanol mixture may be transferred into centrifuge tubes and centrifuged (*e.g.*, 100 x g, for 5 minutes at room temperature) prior to transfer to the 96-well plates for the absorbance determination. If the mixture is cloudy and/or precipitate is noted after the initial plate reading, the sample may be centrifuged after the initial plate reading, added to the 96-well plate, and then the plate re-read. The same centrifugation procedure may be performed after the isopropanol extraction period in the definitive assay.

The absorbance of the test article samples will be determined by subtracting the mean isopropanol blank value from the absorbance of the test article samples. If the OD_{550} of the test article sample is **> 0.08** the test article will be treated as outlined in section §7.12 for the colorant controls procedures.

7.9 Receipt of EpiDerm™ Skin Model (EPI-200)

Upon receipt of the EpiDerm™ Kit, the reagents will be stored as indicated by the manufacturer. The EpiDerm™ tissues will be stored at 2-8°C until used.

On the day of dosing, an appropriate volume of EpiDerm™ Assay Medium will be removed and warmed to approximately 37°C. Nine-tenth (0.9) mL of Assay Medium will be aliquoted into the wells of 6-well plates. Each EpiDerm™ tissue will be inspected for air bubbles between the agarose gel and cell culture insert prior to opening the sealed package. Tissues with air bubbles greater than 50% of the cell culture insert area will not be used. The 24-well shipping containers will be removed from the plastic bag and the surface disinfected with 70% ethanol. An appropriate number of EpiDerm™ tissues will be transferred aseptically from the 24-well shipping containers into the 6-well plates. The EpiDerm™ tissues will be incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air (standard culture conditions) for at least one hour. The medium will be aspirated and 0.9 mL of fresh medium will be added to each assay well below the EpiDerm™ tissue insert. Upon opening the bag, any unused tissues remaining on the shipping agar at the time of tissue transfer will be briefly gassed with an atmosphere of 5% CO_2/95% air, and the bag will be sealed and stored at 2-8°C for subsequent use.

7.10 Skin Corrosion Assay

The test and control articles will be tested by treating four EpiDerm™ tissues per

material. Two tissues will be used to assess viability after the 3-minute exposure and two will be used to assess viability after the 60-minute exposure. Fifty microliters (50 µL) of control or liquid test articles or approximately 25 mg of solid test article (measured using a 25 mg sharp spoon) will be applied to the apical side of the EpiDerm™ tissue. Each EpiDerm™ tissue treated with a solid test article will receive 25 µL of sterile, deionized water applied directly onto the test article. The solid test article will be gently mixed, and spread over the tissue surface using a sterile bulb-headed rod if needed. The 3-minute exposure time will begin as soon as the test article is spread onto the tissue (either directly or with the dosing device). The 3-minute exposure groups will be held at room temperature during the treatment incubation, while the 60-minute exposure groups will be placed in the incubator at standard culture conditions during treatment.

A 10X stock of MTT prepared in PBS (filtered at time of batch preparation) will be thawed and diluted in warm MTT Addition Medium to produce a 1.0 mg/mL solution no more than two hours before use. Three hundred µL of the MTT solution will be added to each designated well of a pre-labelled 24-well plate.

At the end of the test article incubation period, each tissue will be rinsed and transferred to the MTT solution. Rinsing will be accomplished using warm (approximately 37°C) sterile CMF-DPBS delivered from a plastic squeeze bottle. The tip of the bottle shall be cut so as to enlarge the orifice to at least 3 mm (interior diameter) that will allow a gentle stream of CMF-DPBS to be delivered to the surface of the tissue. After the appropriate exposure time, each EpiDerm™ tissue will be thoroughly rinsed on both sides of the tissue insert with CMF-DPBS. The CMF-DPBS will be decanted and the cell culture insert will be blotted on sterile paper towels. The EpiDerm™ tissues will be transferred to the appropriate wells on the pre-labelled 24-well MTT plate after rinsing. The 24-well plates will be incubated at standard culture conditions for 3 ± 0.1 hours.

After 3 ± 0.1 hours, the cell culture insert will be removed from the MTT solution, the bottom blotted on sterile paper towels, cleared of excess liquid, and the cell culture insert transferred to a pre-labelled 24-well plate containing 2.0 mL of isopropanol in each designated well. The plates will be covered with paraffin film and stored in the refrigerator (2-8°C) until all of the tissues have been placed into the isopropanol. If necessary, the plate may be stored sealed overnight before the extraction step is begun.

To extract the reduced MTT, the plates will be shaken for 2 - 3 hours at room temperature. The shaking should be sufficiently vigorous to move the isopropanol around the cell culture insert. At the end of the extraction period, the liquid within the cell culture inserts will be decanted into the well from which the cell culture insert was taken. The extracted MTT solution will be mixed and 200 µL will be transferred to the appropriate wells of a pre-labelled 96-well plate. Two hundred µL of isopropanol will be added to the 2 wells designated as blanks. The absorbance at 550 nm (OD_{550}) of each well will be measured with a Molecular Devices Vmax plate reader with the Automix function on.

7.11 Killed Controls (KC) for Assessment of Residual Test Article Reduction of MTT

In cases where the test article is shown to reduce MTT, only test articles that remain bound to the tissue after rinsing could result in a false MTT reduction signal. To evaluate whether residual test article is binding to the tissue and leading to a false MTT reduction signal, a functional check (using freeze-killed control tissue) is performed using freeze-killed tissue.

Freeze killed tissue is prepared by placing untreated EpiDerm™ tissues in the (-20°C) freezer overnight. The killed tissue will be allowed to thaw at least once at room temperature, and then placed back into the -20°C freezer overnight. Once killed, the tissue may be stored indefinitely in the freezer. Freeze-killed tissues may be received already prepared from MatTek Corporation. Once received at IIVS, the freeze-killed tissues will be warmed to room temperature, and then transferred to the -20°C freezer until use.

To test for residual test article-mediated MTT reduction, two killed tissues will be treated with the positive control or test article concurrently with the viable tissue. All assay procedures will be performed in the same manner as for the viable tissue. Two negative control-treated (50 µL of sterile, deionized water) killed controls will be tested at the same exposure times as the viable negative control-treated tissues, since a small amount of MTT reduction is expected from the residual NADH and associated enzymes within the killed tissue, and will generate some background reduction of MTT.

If little or no MTT reduction is observed in the test article-treated killed control, the MTT reduction observed in the test article-treated viable tissue may be ascribed to the viable cells. If there is appreciable MTT reduction in the treated killed control, additional steps must be taken to account for the chemical reduction or the test article may be considered untestable in this system. The OD_{550} values from the killed controls will be analyzed as described in **§7.14**.

7.12 Colorant Controls (CC) for Assessment of MTT Interaction

In cases where a test article is shown to interfere with the MTT measurement (see §7.8 *Assessment of Colored or Staining Materials*), colorant controls (CC) will be tested. **Ideally, the colorant controls will be tested concurrently with the definitive assay; however, in some cases (*e.g.*, insufficient tissues) follow-up testing of the colorant controls on a separate testing date may be warranted.** To determine whether the test article is interfering with the MTT measurement, at least one live colorant control (CC) tissue will be tested per test article. The CC tissues will be treated in the same manner as the live tissues with the exception that the tissues will incubated in 0.3 mL of Maintenance Medium instead of MTT for 3 ± 0.1 hours at standard culture conditions. After the 3 ± 0.1 hours incubation period, the tissues will be processed as outlined in section 7.10.

The CCs will be analyzed as described in §7.14.

7.13 Colorant Control-Killed Control (CC-KC) for Assessment of MTT Interaction

In cases where a test article is shown to reduce MTT **and** also interfere with the MTT measurement, colorant control-killed controls (CC-KC) will be tested concurrently. The CC-KC will be tested to avoid a possible double correction for color interference. Two killed control tissues per test article and per exposure time will be used. The CC-KC tissues will be treated in the same way as the CC tissues (i.e. incubation in 0.3 mL of Maintenance Medium instead of MTT for 3 ± 0.1 hours at standard culture conditions). After the 3 ± 0.1 hours incubation period, the tissues will be processed as outlined in section 7.10.

The CC-KC will be analyzed as described in §7.14.

7.14 Presentation of Data

Generally, all calculations will be performed using an Excel spreadsheet. The raw absorbance values will be captured, and the following calculations made:

The mean OD_{550} value of the blank wells will be calculated. The corrected mean OD_{550} value of the negative control, positive control, and test article(s) will be determined by subtracting the mean OD_{550} value of the blank wells from the mean OD_{550} values. In general, the average of the corrected mean OD_{550} values of the 3-minute and 60-minute negative controls will be calculated for subsequent % of Control viability calculations.

Corrected OD_{550} = Test or control article exposure time OD_{550} – Blank mean OD_{550}

When applicable, the final corrected OD_{550} of the test article(s) will be determined using OD_{550} values for the killed controls (KC), colorant controls (CC), and/or colorant control-killed controls (CC-KC). The final corrected OD_{550} determination will be used to determine relative viability. *Note: any negative value that may be obtained for KC, CC or CC-KC will be equaled to zero for calculation purposes.*

If killed controls (KC) are used: the following additional calculations will be performed to correct for the amount of MTT reduced directly by positive control or test article residues. The mean raw OD_{550} value for the negative control killed control will be subtracted from the mean raw OD_{550} values for each of the positive control or test article-treated killed controls (at each appropriate exposure time), to determine the net OD_{550} values (KC OD_{550}) of the positive control- or test article-treated killed controls.

Net OD_{550} for each test article KC = Mean Raw OD_{550} test article KC – Mean Raw OD_{550} negative control KC

The net OD_{550} values represent the amount of reduced MTT due to direct reduction by test article residues. The net amount of MTT reduction will be subtracted from the corrected OD_{550} values of the viable treated tissues to obtain a final corrected OD_{550} value. Although the algorithms discussed are performed to calculate the final endpoint analysis at the treatment group level, the same calculations can be applied to the individual replicates.

KC-Corrected test article exposure time OD_{550} = Corrected test article exposure time OD_{550} – KC OD_{550}

If colorant controls (CC) are used, the OD_{550} value of the CC (CC OD_{550}) will be subtracted from the corrected OD_{550} values of the viable treated tissues to obtain a final corrected OD_{550} value.

CC-Corrected test article exposure time OD_{550} = Corrected test article exposure time OD_{550} – CC OD_{550}

If colorant control-killed controls (CC-KC) are used, the OD_{550} value of the CC-KC will be added to corrected OD_{550} values of the viable treated tissues to obtain a final corrected OD_{550} value.

CC-KC-Corrected test article exposure time OD_{550} = Corrected test article exposure time OD_{550} + CC-KC OD_{550}

The corrected OD_{550} value(s) of the test article(s) for the KC, CC, and/or CC-KC will be used to determine the final corrected OD_{550} value.

Final Corrected OD_{550} of the test article exposure time =
Corrected test article exposure time OD_{550} – KC OD_{550} - CC OD_{550} + CC-KC OD_{550}

The individual viability values will be calculated as follows:

$$\% \text{ Viability} = \frac{\text{Final corrected } OD_{550} \text{ of Test Substance or Positive Control Exposure time}}{\text{Average corrected mean } OD_{550} \text{ of the 3-minute and 60-minute of Negative Controls}} \times 100$$

The standard deviation (SD) of the tissue viability will then be calculated. The following % Viability (% of Control) calculations will be made:

$$\% \text{ Viability} = \frac{\text{Final corrected mean } OD_{550} \text{ of Test Substance or Positive Control Exposure time}}{\text{Average corrected mean } OD_{550} \text{ of the 3-minute and 60-minute of Negative Controls}} \times 100$$

Finally, the upper limit and lower limit range of tissue viability will be calculated as follows:

Upper limit = % Viability (mean) of test substance or positive control exposure time + (2 x SD)
Lower limit = % Viability (mean) of test substance or positive control exposure time – (2 x SD)

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The assay will be accepted if the following criteria are met:

1) the positive control results in a corrosive classification (i.e., <50% cell viability compared to negative controls, after a 3-minute exposure, and/or <15% after a 60-minute exposure).

2) the mean OD_{550} value of the negative control tissues is ≥ 0.8 and < 2.8.

11.0 RECORDS AND ARCHIVES

A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data, reports and specimens will be retained in the archives for a period of either a) 5 years, b) the length of time specified in the contract terms and conditions, or c) as long as the quality of the preparation affords evaluation, whichever is applicable.

12.0 TEST MATERIAL RETENTION

Unless indicated otherwise, all test articles provided by the sponsor and dose solutions used for testing or analysis before or during the course of the assay will be retained for one year after completion of the final report. These test articles and dose solutions may be disposed after this 1 year retention period according to IIVS SOP.

13.0 PROTOCOL AMENDMENTS

When it becomes necessary to change the approved protocol for a specific study, the change and the reason for it should be put in writing and signed by the Study Director as soon as practical. When the change may impact the study design and/or execution, verbal agreement to make this change should be made between the Study Director and Sponsor. This document is then provided to the Sponsor and is attached to the protocol as an amendment.

14.0 REFERENCES

Cannon, C.L., Neal, P.J., Kubilus, J., Klausner, M., Swartendrubber, D.C., Squire, C.A., Kremer, M.J., and Wertz, P.W. (1994) Lipid and ultrastructural characterization of a new epidermal model shows good correspondence to normal human skin. J. Invest. Derm. 102(4):600.

Harbell, J.W., Southee, J.A., Cannon, C.L., Neal, P.J., Kubilus, J., and Klausner, M. (1994) Inter- and intralaboratory reproducibility of a three dimensional human epidermal model - EpiDerm™. The Toxicologist 14(1):108.

Barratt, M.D., Brantom, P.G., Fentem, J.H., Gerner, I., Walker, A.P., and Worth, A.P. (1998) The ECVAM international validation study on in vitro tests for skin corrosion. 1. Selection and distribution of the test chemicals. Toxicol. In Vitro 12:471-482.

Fentem, J.H., Archer, G.E.B., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Esdaile, D.L., Holzhutter, H.G., and Liebsch, M. (1998) The ECVAM international validation study on in vitro tests for skin corrosivity. 2. Results and evaluation by the Management Team. Toxicol. In Vitro 12:483-524.

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MatTek Corporation. Protocol for: IN VITRO EpiDerm™ SKIN CORROSION TEST (EPI-200-SCT) for use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm. SOP MK-24-007-0024, Rev. 03/29/2012.

OECD GUIDELINES FOR THE TESTING OF CHEMICALS. Test Guideline 431 "*In vitro* skin corrosion: reconstructed human epidermis (RHE) test method", Adopted 29 July 2016.

15.0 APPROVAL

_____ 20 April 2018
DATE

_____ 20 April 2018
IIVS STUDY DIRECTOR DATE

PROTOCOL ATTACHMENT 1

IIVS Test Article Designation	Sponsor Designation
18AD37	

Test Article Preparation: The test article(s) will be tested neat.

REGULATORY REQUIREMENTS:

Will this study be conducted according to **GLPs**? [**X**] **YES** or [ ] **NO**

If **YES**, please indicate which agency(ies) guidelines are to be followed:
[ ] FDA; [**X**] EPA TSCA; [ ] EPA FIFRA; [ ] OECD; [ ] Other

AMENDMENT(S):

1) Location: Protocol Attachment 1

Amendment: **Change**:

"Test Article Preparation: The test article(s) will be tested neat."

To:

"Test Article Handling and/or Preparation:

The test article will be tested neat. The test article was provided in a liquid nitrogen pressurized cylinder. Before being use in the step(s) of the assay, an aliquot of the test article needed for the assay will be removed from the cylinder, placed into a glass vial labeled according to the SOP(s) of the Testing Facility, and stored in the same conditions (15°C - 30°C) as the original container received from the Sponsor. After use, the remaining aliquoted sample will be discarded according to the disposal instruction in the Safety Data Sheet (SDS) provided by the Sponsor. The exact amount of the aliquoted test article and any specific aspects of the handling procedures will be captured in the study workbook and report.

The tissues treated with the test article (for each of the exposure times of 3 and 60 minutes, respectively) will be cultured on plates separated from the assay controls and from each other to avoid any contamination through possible evaporation that may occur.

Reason: Protocol generation error.

APPROVAL:

STUDY DIRECTOR — 30 April 2018 DATE

APPENDIX B

EPIDERM ™ CORROSIVITY BIOASSAY

Study No. 18AD37.050054
Plate: 1
Read Date: 5/4/2018
Read By: GEC

Plate Map:

	1	2	3	4	5	6	7	8	9	10	11	12
A:	CTRL 60 min	CTRL 60 min	PCC1	PCC1	KCNeg60	KCNeg60	KCPCC1	KCPCC1				
B:	CTRL 3 min	CTRL 3 min	PCC2	PCC2	KCNeg3	KCNeg3	KCPCC2	KCPCC2	KCPCC2-second set			
C:			T2C1	T2C1								
D:			T2C2	T2C2								
E:												
F:												
G:		BLNK										
H:		BLNK										

Raw Data: Optical Density (OD_{550})

	1	2	3	4	5	6	7	8	9	10	11	12
A:	1.889	1.756	0.294	0.389	0.108	0.150	0.241	0.159				
B:	1.909	1.833	0.498	0.471	0.125	0.137	0.341	0.115	0 339	0.411		
C:			1.947	1.932								
D:			2.030	1.833								
E:												
F:												
G:		0.035										
H:		0.035										

Results:

Average Corrected Control OD: CTRL 60 min 1.788 Average Blank OD: 0.035
CTRL 3 min 1.836 Average 60 min and 3 min CTRL: 1.812

	Trial	Map Prefix	Exposure Time (minutes)	Corrected OD_{550} Values Individual Values		KC Corrected OD_{550} Values * Individual Values		Mean	Individual Viability Values		SD of Viability	Range of Tissue Viability		%Viability (Mean)	Corrosivity	Sub-Category
				1	2	1	2					Upper limit	Lower limit			
Positive Control		PCC1	60	0.259	0.354	0.188	0.283	0.236	0.4%	15.6%	3.7%	20.4%	5.6%	13.0%	corrosive	1A
8N KOH		PCC2	3	0.463	0.436	0.230	0.203	0.217	12.7%	11.2%	1.1%	14.1%	9.9%	12.0%	corrosive	
Test Article	B1	T2C1	60	1.912	1.897			1.905	105.5%	104.7%	0.6%	106.3%	103.9%	105.1%	non-corrosive	
18AD37		T2C2	3	1.995	1.798			1.897	110.1%	99.2%	7.7%	120.1%	89.3%	04.7%	non-corrosive	

Killed Control Adjustments

Negative killed 60 minute control = 0.129
Negative killed 3 minute control = 0.131

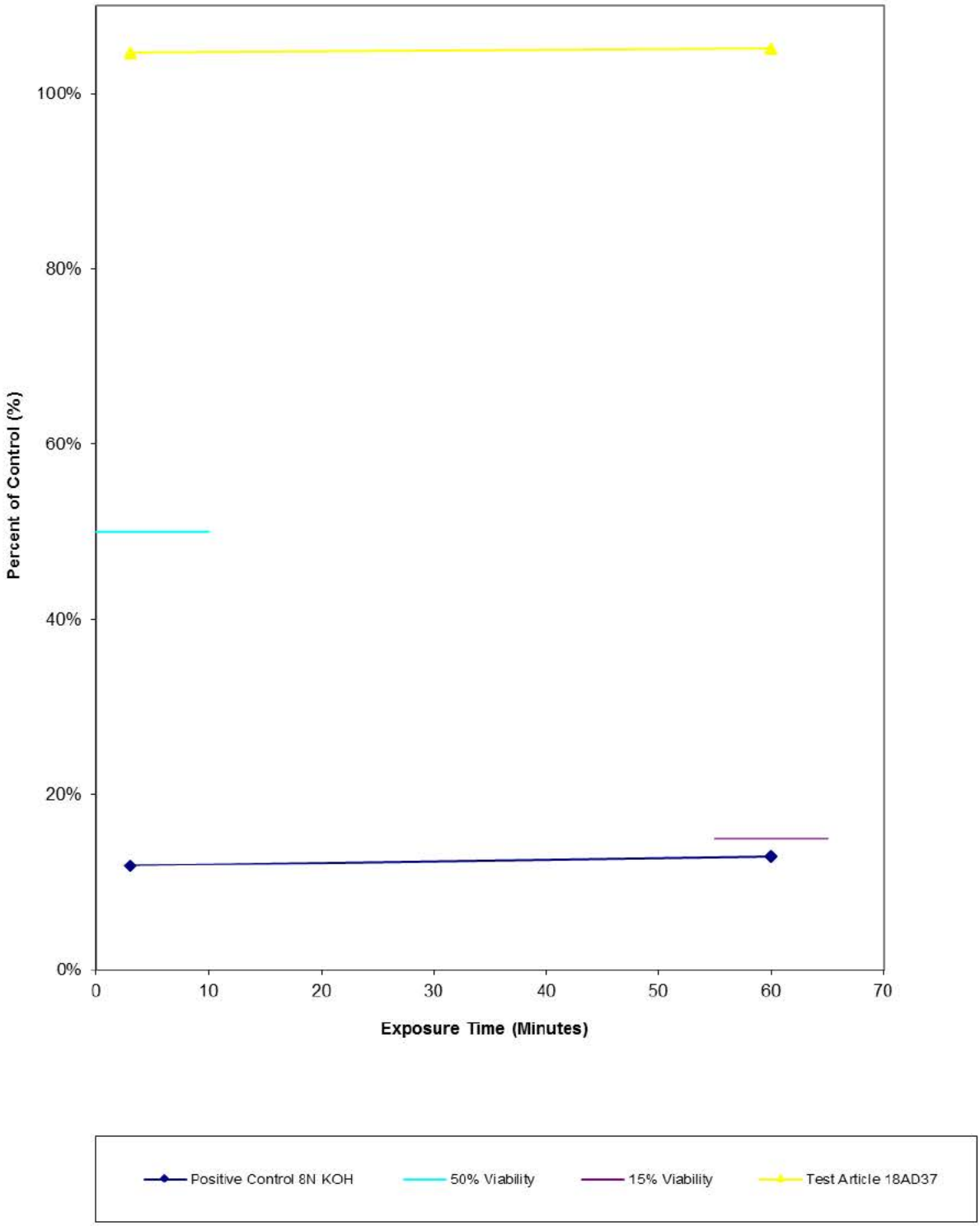
Test Article		KC Raw OD				KC Net OD (Raw TA KC OD-Raw Neg KC OD)				Mean
Positive Control	60 min	0.241	0.159	NA	NA	0.112	0.030	NA	NA	0.071
	3 min	0.341	0.115	0.339	0.411	0.2 0	-0.0 6	0.208	0.280	0.233

value equaled to zero per protocol

KCPCC2-second set: conducted because the reduction of MTT by the second killed control tissue treated with the assay positive control was visibly less compared to the reduction by the tissue replicate.

B1

EpiDerm™ Corrosivity Assay



COLORANT CONTROLS ASSESSMENT

Study No. 18AD37 050054
Plate: 1
Read Date: 05/01/18 Read By: EW
Raw Data: Optical Density (OD_{550})

	1	2	3	4	5	6	7	8	9	10	11	12
A:	0.036	0.034										
B:												
C:												
D:												
E:												
F:												
G:												0 033
H:												0 034

Plate Map:

	1	2	3	4	5	6	7	8	9	10	11	12
A:	T1C1	T1C1										
B:												
C:												
D:												
E:												
F:												
G:												BLNK
H:												BLNK

Average Blank OD: 0.034

Test Article	Trial	Map Prefix	Average OD_{550} Values	Corrected OD_{550} (Avg. TA- Avg. Blank OD)	Possible MTT Interference? (Corrected OD550>0 08)
18AD37	B1	T1C1	0.035	0 002	No

APPENDIX C

C2

SIGMA-ALDRICH®

sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Potassium hydroxide solution - volumetric, 8.0 M KOH (8.0N)

Product Number:	**P4494**
Batch Number:	**SLBM9898V**
Brand:	SIAL
MDL Number:	MFCD00003553
Formula:	HKO
Formula Weight:	56.11 g/mol
Quality Release Date:	07 APR 2015

KOH

Test	Specification	Result
Appearance (Turbidity)	Clear	Clear
Appearance (Color)	Colorless	Colorless
Appearance (Form)	Liquid	Liquid
Normality by HCl Titration	7.92 - 8.08 N	7.92 N

Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri US

Quality Biological

MAKING SOLUTIONS WORK

PRODUCT: WATER – Ultra Pure (Cell Culture Grade)

CATALOG#: 118-162-101 Size: 500ml

LOT#: 721803

CERTIFICATE OF ANALYSIS

This product is de-ionized by reverse osmosis and sterile filtered.

	Specifications	Result
RESISTIVITY:	15 – 20 Meg Ohms/cm	17.90 Meg Ohms/cm
ENDOTOXIN:	Less than 0.06 Eu/ml	pass
APPEARANCE:	Clear colorless fluid.	pass
STERILITY:	No evidence of bacterial or fungal contamination after 14 days testing on Fluid Thioglycollate and Tryptic Soy Broth.	pass

RECOMMENDED STORAGE CONDITIONS: 15 – 30°C

MANUFACTURED DATE: 08/2016

EXPIRATION DATE: 08/2018

10/7/2016

Quality Assurance Date

FOR RESEARCH USE ONLY
NOT FOR IN-VITRO DIAGNOSTIC USE.

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Email: customerservice@qualitybiological.com • Web Site: www.qualitybiological.com

Report Title

Composition and Spectral Confirmation of

Date Study Completed

May 15, 2018

Table of Contents

Certification

We, the undersigned, declare that this report provides an accurate record of the procedure and results.

Report submitted by:

07/30/18
Date

30 July 2018
Date

Composition and Spectral Confirmation of

1.0 Introduction

This report presents the composition and spectral confirmation of This report also contains summaries of the analytical procedures employed to determine the composition and to identify the impurities of ncluding GC/MS, GC/FID, NMR, FT-IR spectroscopy, UV/Vis spectroscopy.

1.1 Product Identity and Composition

Test Compound

2.0 Test Item

A representative sample of was used as a test item in this study. Unique identification information is listed in Table 1.

3.0 Experimental

Standard and sample solutions were analyzed by using the following analytical procedures:

3.1 GC/MS Procedure for the Determination of Composition of

Scan mode GC/MS chromatograms of are presented in Figures 1-3 with mass spectra of components (Figures 4-8). GC-FID chromatograms of are presented in Figures 9-12 along with area counts and percentages represented in Table 2-3. The GC method parameters are listed below along with the procedure for the preparation of amples. The data was processed using Agilent Chemstation.

Operating Conditions

Gas Chromatograph: Agilent 7890A GC system
Syringe Size: 10 µL
Injection Volume: 1 µL
Rinse Solvent: Vertrel™ XF
Detector: 5975C MSD with Triple –Axis Detector
MS Source Temperature: 230°C
MS Quad Temperature: 150°C
Mass Scan Range: 40 - 800 amu
MS and FID Columns: RTX-1: 105 m x 250 µm x 1.0 µm
Solvent Delay: 0 min.
FID Hydrogen Flow: 40 mL/min
Air Flow: 400 mL/min
Make up Gas Flow: 25 mL/min
Make up Gas Type: Helium
Column: RTX-1: 105 m x 250 µm x 1.0 µm
Column Gas Type: Helium
Gas Flow Rate: 1.2 mL/min
Flow Mode: Constant Flow
Inlet Temperature: 250°C
Mode: Split
Split Ratio: 50:1
Standard GC/MS and GC/FID Conditions:
Oven Initial Temperature: 35°C
Initial Hold Time: 15 min
Ramp 1 Rate: 15°C/min
Ramp 1 Value: 100°C
Ramp 1 Hold Time: 5 min
Ramp 2 Rate: 20°C/min
Ramp 2 Value: 280°C
Ramp 2 Hold Time: 5 min
Oven Final Temperature: 280°C
Oven Final Time: 38.333 min
Cryo-GC/FID Conditions:
Oven Initial Temperature: 0°C
Initial Hold Time: 10 min
Ramp 1 Rate: 5°C/min
Ramp 1 Value: 100°C

Ramp 1 Hold Time: 5 min
Ramp 2 Rate: 20°C/min
Ramp 2 Value: 280°C
Ramp 2 Hold Time: 0 min
Oven Final Temperature: 280°C
Oven Final Time: 44.000 min
Cryo: On
Cryo Use Temperature: 0°C

Sample Preparation:

A stainless-steel gas cylinder containing was fitted with 1/8" PFA tubing using a brass nut which was then connected to a glass three-way "T" adaptor plumbed to a 1L Tedlar™ gas bag, and a vacuum manifold. Tubing connect the "T" adaptor to the Tedlar™ gas bag was PFA while tubing connecting the "T" adaptor to the vacuum manifold was polyethylene. Once all components were connected, the system was brought to 0 barr with the vacuum manifold for 10 minutes. After this period, the system was then brought to 758 barr with dry nitrogen directly from the vacuum manifold. This cycle was repeated two more times. Once an inert transfer atmosphere was maintained, th ylinder was positioned in a vertical manner above the Tedlar™ bag with the valve on the bottom so that liquid travelled directly to the Tedlar™ bag upon opening. The valve on the cylinder was gently cracked to transfer portion-wise to the Tedlar™ gas bag. Once approximately 5 mL of liquid was transferred to the bag all valves were closed, the system was disassembled, and all inlets and valves were purged with a stream of high pressure air within the fume hood to ensure that any residual was removed. The was analyzed by GC/MS and GC/FID as a neat solution with 1 µL injections and high split ratios.

3.2 Procedure for Generation of an NMR Spectrum of

NMR spectra are presented in Figures 14-25.

Operating Conditions:

The ^{1}H, ^{13}C, and ^{19}F spectra were acquired on a 400 MHz Varian DirectDrive® Spectrometer equipped with a 5mm H,F {C/P}inverse triple resonance probe at 25°C. Spectra were processed using ACD Labs Spectrus Processor software.

Proton (^{1}H) Acquisition Parameters:

Spectrometer Frequency: 399.906 MHz
Pulse Program: s2pul
Number of Acquisitions: 32
Relaxation Delay: 20.00 s
Spectral Width: 6410.3 Hz
Pulse: 30°
Transmitter Offset: 6.175 ppm
Acquisition Time: 2.556 s

Temperature: 26°C

Proton Processing Parameters
Total Processed Data Points/Zero Filling: 32768
Line Broadening: 0.30 Hz
Proton chemical shifts were referenced to TMS at 0.00 ppm.

Carbon (^{13}C), ^{1}H decoupled, Acquisition Parameters:
Spectrometer Frequency: 125.6572 MHz
Pulse Program: s2pul
Receiver Gain: 60.00
Number of Acquisitions: 640
Relaxation Delay: 1.00 s
Spectral Width: 34721.69 Hz
Transmitter Offset: 15078.9785 Hz
Acquisition Time: 1.0486 s
Temperature: 25°C

Carbon, ^{1}H decoupled, Processing Parameters:
Data points: 65536
Line broadening: 0.50 Hz
Carbon spectrum was referenced to TMS at 0.00 ppm.

Fluorine (^{19}F) Acquisition Parameters:
Spectrometer Frequency: 376.61 MHz
Pulse Program: zgflqn
Pulse: 90°
Number of Acquisitions: 64
Relaxation Delay: 20.00 s
Spectral Width: 237.10 Hz
Transmitter Offset: -84.22 ppm
Acquisition Time: 1.80 s
Temperature: 25°C

Fluorine Processing Parameters:
Data points: 65536
Line broadening: 0.30 Hz

Sample Preparation:
A 5mm borosilicate NMR tube was connected to a vacuum manifold adaptor and placed under an inert atmosphere on a vacuum manifold using dry nitrogen. 0.8 mL of $CDCl_3$ was added to the NMR under a nitrogen flow, followed by 5 µL of

The tube was sealed with a PTFE cap under nitrogen flow and NMR measurements were taken thereafter.

3.3 *Procedure for Generation of an FT-IR Spectrum for*

A representative IR spectrum is presented in Figure 26.

Operating Conditions

Instrument: Nexus 470
Resolution: 4 cm^{-1}
Number of Scans: 32
Velocity: 0.6329 cm s^{-1}
Beamsplitter: KBr
Detector Type: deuterated triglycine sulfate (DTGS) with KBr windows
Data Spacing: 1.928 cm^{-1}
Range: 400 - 4000 cm^{-1}
Background: Nitrogen
Gas cell path length: 10 cm
Gas cell windows: KBr

Preparation of Sample

An IR gas cell was connected to a vacuum manifold adaptor and placed under an inert atmosphere on a vacuum manifold using dry nitrogen. After taking a background of the nitrogen, 5 µL of was added to the gas cell under a nitrogen flow and analyzed over the range of 400 cm^{-1} to 4000 cm^{-1}.

3.4 *Procedure for Generation of a UV-Vis Spectrum for*

A representative UV/Vis spectrum is presented in Figure 27.

Operating Conditions

Instrument: B&W Tek Exemplar® LS spectrometer
Range: 200-850 nm
Data Interval: 0.4 nm
Integration Time: 500 ms
Lamps: deuterium (200-370 nm), tungsten halide (370-850 nm)
Baseline Correction: CH_2Cl_2 used as background scan
Solvent: CH_2Cl_2
Cuvette Type: Quartz cuvette

Preparation of Sample Solution and Spectra Collection

A quartz cuvette with a J. Young fitting was connected to a vacuum manifold adaptor and placed under an inert atmosphere on a vacuum manifold using dry nitrogen. CH_2Cl_2 was added to the cuvette under a nitrogen flow, and a background/reference scan was obtained. After, was added to the cuvette, also under nitrogen. The spectrum was taken over the range of 200 nm to 850 nm.

4.0 Results and Discussion

Appendix A – Tables

CBI SUBSTANTIATION
For PMN, SNUN, TMEA, LVE, and LOREX filings

Use of this form is recommended, but not required.
Choose an item.

Technical Contact Name:
Technical Contact Phone Number:
Technical Contact Email Address:
Submitting Company Name
Submission Number (if known):

***Important**: You are responsible for substantiating **each** data element you claim as CBI unless that item is exempt from the substantiation requirement according to TSCA section 14(c)(2). This template identifies the information that EPA considers to generally be exempt in this submission type. EPA expects that it will generally deny non-exempt CBI claims that are not substantiated, so recommends that the submitter carefully review the TSCA submission to assure that the substantiation addresses all CBI claims that require substantiation. The substantiation must also clearly indicate which CBI claim(s) it is intended to cover. In this template, similar data elements have been grouped together to permit substantiation of multiple such elements at one time.*

EPA expects that some types of CBI claims will be more difficult to support than others or are in some cases restricted by TSCA or its implementing regulations. Health and safety studies and information from such studies, with limited exceptions, may not be withheld by EPA as confidential. For more information, please visit: https://www.epa.gov/tsca-cbi/what-include-cbi-substantiations

40 CFR § 2.208 specifies the substantive criteria that are used to determine whether information is entitled to confidential treatment. Among these criteria is the substantial competitive harm(s) that would be caused by public disclosure of the information that you have claimed as CBI. Failure to sufficiently explain this harm in the substantiation for any data element claimed as CBI may result in a denial of the CBI claim for that data element.

The "CBI Claim" column on this substantiation document is intended to be used to indicate that a CBI claim has been made for the corresponding information in the PMN form. Please verify that the information you indicate is claimed as CBI on this substantiation document is in fact claimed as CBI in the PMN form and/or attachments. This template cannot be used to assert a CBI claim that is not asserted in the PMN form.

I. COMPETITIVE HARM QUESTIONS

Part I Section A Submitter Identification	**CBI Claim**	**Substantiation** *(Explain how public disclosure of this information is likely to cause substantial harm to your business's competitive position.)*
Signature and Date of Authorized Official (Page 2)	☑	Disclosure of the submitter would permit a competitor to specifically know that the submitter is seeking develop new products for the generic use disclosed in the PMN. This would result in harmful effects on submitter's competitive advantage.
Signature and Date of Agent (Page 2)	☐	
Person Submitting Notice (Part I Section A.1.a)	☑	
Agent (Part I Section A.1.b)	☐	
Joint Submitter (Part I Section A.1.c)	☐	Click or tap here to enter text.
Technical Contact (Part I Section A.2)	☑	Disclosure of the submitter would permit a competitor to specifically know that the submitter is seeking develop new products for the generic use disclosed in the PMN. This would result in harmful effects on submitter's competitive advantage.
Pre-notice Communication (PC) (Part I Section A.3)	☐	Click or tap here to enter text.
Previously Submitted Exemption Application (Part I Section A.4)	☐	
Previously Submitted Bona Fide (Part I Section A.5)	☐	
Part I Section B Chemical Identity Information	**CBI Claim**	**Substantiation** *(Explain how public disclosure of this information is likely to cause substantial harm to your business's competitive position.)*
Chemical Class (Part I Section B.1.a)	☑	Disclosure of information about substance identity would permit a competitor to specifically know that the submitter is seeking develop a specific type of new product for the generic use disclosed in the PMN. This would result in harmful effects on submitter's competitive advantage.

Chemical Name (Part I Section B.1.b)	☑	Exempt prior to the substance first being offered for commercial distribution – for PMN, LVE, TMEA, and LOREX submissions, no substantiation is required for CBI claims on this specific data element. Submitters of SNUNs and requests to modify an LVE or LOREX may not be exempt from this substantiation requirement. PMN substance has not been offered for commercial distribution. Therefore, substantiation is not required at this time.
Molecular Formula (Part I Section B.1.d)	☑	Exempt prior to the substance first being offered for commercial distribution – for PMN, LVE, TMEA, and LOREX submissions, no substantiation is required for CBI claims on this specific data element. Submitters of SNUNs and requests to modify an LVE or LOREX may not be exempt from this substantiation requirement. PMN substance has not been offered for commercial distribution. Therefore, substantiation is not required at this time.
Chemical Structure Diagram for Class I (Part I Section B.1.e)	☑	Exempt prior to the substance first being offered for commercial distribution – for PMN, LVE, TMEA, and LOREX submissions, no substantiation is required for CBI claims on this specific data element. Submitters of SNUNs and requests to modify an LVE or LOREX may not be exempt from this substantiation requirement. PMN substance has not been offered for commercial distribution. Therefore, substantiation is not required at this time.
Precursor Substances Class II (Part I Section B.1.e.1)	☑	Exempt – No substantiation required for this specific data element claim
Reaction or Process for Class II (Part I Section B.1.e.2)	☑	Exempt – No substantiation required for this specific data element claim
Range of Composition and Typical Composition for Class II (Part I Section B.1.e.3)	☑	Exempt – No substantiation required for this specific data element claim
Polymer Information (Part I Section B.2.a)	☑	Exempt prior to the substance first being offered for commercial distribution – for PMN, LVE, TMEA, and LOREX submissions, no substantiation is required for CBI claims on this specific data element. Submitters of SNUNs and requests to modify an LVE or LOREX may not be exempt from this substantiation requirement. PMN substance has not been offered for commercial distribution. Therefore, substantiation is not required at this time.

Monomer or Other Reactant Specific Chemical Name (Part I Section B.2.b.1)	☑	Exempt – No substantiation required for this specific data element claim
Monomer or Other Reactant Specific Chemical Name Typical Composition (Part I Section B.2.b.3)	☑	Exempt – No substantiation required for this specific data element claim
Monomer or Other Reactant Specific Chemical Name Include in Identity (Part I Section B.2.b.4)	☑	Exempt – No substantiation required for this specific data element claim
Monomer or Other Reactant Specific Chemical Name Max Residual (Part I Section B.2.b.6)	☑	Exempt – No substantiation required for this specific data element claim
Current Chemical Abstracts (CA) Name and Number for Polymer (Part I Section B.2.d)	☑	Exempt prior to the substance first being offered for commercial distribution – for PMN, LVE, TMEA, and LOREX submissions, no substantiation is required for CBI claims on this specific data element. Submitters of SNUNs and requests to modify an LVE or LOREX may not be exempt from this substantiation requirement. PMN substance has not been offered for commercial distribution. Therefore, substantiation is not required at this time.
Chemical Structure Diagram (Part I Section B.2.e)	☑	Exempt prior to the substance first being offered for commercial distribution – for PMN, LVE, TMEA, and LOREX submissions, no substantiation is required for CBI claims on this specific data element. Submitters of SNUNs and requests to modify an LVE or LOREX may not be exempt from this substantiation requirement. PMN substance has not been offered for commercial distribution. Therefore, substantiation is not required at this time.
Impurities (Part I Section B.3)	☑	Exempt – No substantiation required for this specific data element claim
Synonyms (Part I Section B.4)	☑	Exempt prior to the substance first being offered for commercial distribution – for PMN, LVE, TMEA, and LOREX submissions, no substantiation is required for CBI claims on this specific data element. Submitters of SNUNs and requests to modify an LVE or LOREX may not be exempt from this substantiation requirement. PMN substance has not been offered for commercial distribution. Therefore, substantiation is not required at this time.
Trade Identification (Part I Section B.5)	☑	Exempt prior to the substance first being offered for commercial distribution – for PMN, LVE, TMEA, and LOREX submissions, no substantiation is required for CBI claims on this specific data

		element. Submitters of SNUNs and requests to modify an LVE or LOREX may not be exempt from this substantiation requirement. PMN substance has not been offered for commercial distribution. Therefore, substantiation is not required at this time.
Byproducts (Part I Section B.7)	☑	Exempt – No substantiation required for this specific data element claim
Part I Section C. Production, Import and Use Information	**CBI Claim**	**Substantiation** *(Explain how public disclosure of this information is likely to cause substantial harm to your business's competitive position.)*
Production Volume (Part I Section C.1)	☑	Exempt – No substantiation required for this specific data element claim
Category of Use (Part I Section C.2.a.1)	☑	Exempt – No substantiation required for this specific data element claim
Use Production (Part I Section C.2.a.4)	☑	Exempt – No substantiation required for this specific data element claim
% in Formulation (Part I Section C.2.a.6)	☑	Exempt – No substantiation required for this specific data element claim
% of Substance Expected Per Use (Part I Section C.2.a.8)	☑	Exempt – No substantiation required for this specific data element claim
Part II Section A Industrial Sites Controlled by the Submitter	**CBI Claim**	**Substantiation** *(Explain how public disclosure of this information is likely to cause substantial harm to your business's competitive position.)*
Site Identity (Part II Section A.1.a) *Provide a detailed substantiation explaining how/why the release of this data element information will substantially harm your company.*	☐	Disclosure of the site identity would reveal the identity of the submitter. Revealing such information would permit a competitor to specifically know that the submitter is seeking develop new products for the generic use disclosed in the PMN. This would result in harmful effects on submitter's competitive advantage.
Number of Sites (Part II Section A.1.a) *Provide a detailed substantiation explaining how/why the release of this data element information will substantially harm your company.*	☑	Disclosure of the number of sites would reveal scope of the manufacturer's domestic activity. Revealing such information would permit a competitor to specifically know that the submitter is seeking develop new products for the generic use disclosed in the PMN and the full scope of the resources being devoted to the activity. This would result in harmful effects on submitter's competitive advantage.
Site Operations (Part II Section A.1.b) *Provide a detailed substantiation explaining how/why the release of this data element information will substantially harm your company.*	☐	Disclosure of the type of activity at the site would reveal the submitter is investing resources at a manufacturing site to introduce new products to market. Revealing such information would permit a competitor to specifically know that the submitter is seeking develop new products for the generic use disclosed in the PMN and the full scope of the

		resources being devoted to the activity. This would result in harmful effects on submitter's competitive advantage.
Amount and Duration (Part II Section A.1.c)	☑	Exempt – No substantiation required for this specific data element claim
Process Description (Part II Section A.1.d)	☑	Exempt – No substantiation required for this specific data element claim
Worker Activity (Part II Section A.2.1) *Provide a detailed substantiation explaining how/why the release of this data element information will substantially harm your company.*	☑	Disclosure of claimed CBI would result in harmful effects on submitter's competitive position. More specifically, disclosure of the specific "worker activity" at our manufacturing site would disclose competitive intelligence information to our competitors regarding process/volume information in the manufacture of the PMN substance (i.e., Equipment Cleaning Losses from a Single, Small Vessel).
Physical Form(s) & % New Substance (Part II Section A.2.5)	☑	Disclosure of claimed CBI would result in harmful effects on submitter's competitive position. Disclosure of this information would enable competitors to gain an understanding the type of formulation being manufactured.
# of Workers Exposed (Part II Section A.2.8) *Provide a detailed substantiation explaining how/why the release of this data element information will substantially harm your company.*	☑	Disclosure claimed CBI would result in harmful effects on submitter's competitive position. Disclosure of the CBI would enable competitors to gain an understanding of the size of our manufacturing process and potentially the length of time we run (i.e., one shift versus entire day).
Maximum Duration (Part II Section A.2.10-11)	☑	Disclosure claimed CBI would result in harmful effects on submitter's competitive position. Disclosure of the CBI would enable competitors to gain an understanding of the size of our manufacturing process and potentially the length of time we run (i.e., one shift versus entire day).
Release Number and Amount of New Substance Released (Part II Section A.3.1-2)	☑	Disclosure of this CBI would reveal information about process efficiency, number of steps in the process, and types of activity in the manufacturing process. This would result in harmful effects on submitter's competitive advantage.
Medium of Release and Control Technology and Efficiency (Part II Section A.3.4-5)	☑	
Destinations of Releases to Water (Part II Section A.3.7)	☑	

Part II Section B Industrial Sites Controlled by Others	**CBI Claim**	**Substantiation** *(Explain how public disclosure of this information is likely to cause substantial harm to your business's competitive position.)*
Operation Description (Part II Section B.1)	☐	Exempt – No substantiation required for this specific data element claim
Letter of Activity and # of Workers Exposed (Part II Section B.2.1-2) *Provide a detailed substantiation explaining how/why the release of this data element information will substantially harm your company.*	☐	Click or tap here to enter text.
Duration of Exposure (Part II Section B.2.4) *Provide a detailed substantiation explaining how/why the release of this data element information will substantially harm your company.*	☐	Click or tap here to enter text.
Protective Equipment/Engineering Controls/Physical Form/ % New Substance/% in Formulation (Part II Section B.2.6-7)	☐	Click or tap here to enter text.
Release Number and Amount of New Substance Released (Part II Section B.2.9-10)	☐	
Media of Release & Control Technology (Part II Section B.2.12)	☐	
Byproducts (Part II Section B.2.14)	☐	
Optional Pollution Prevention Information	**CBI Claim**	**Substantiation** *(Explain how public disclosure of this information is likely to cause substantial harm to your business's competitive position.)*
Pollution Prevention Information (PMN page 11, Form page 16)	☑	Disclosure of this information would reveal specific information about the use and applications of the PMN substance. Revealing such information would permit a competitor to specifically know that the submitter is seeking develop new products for the generic use disclosed in the PMN. This would result in harmful effects on submitter's competitive advantage. We do not want our competitors to know the specific end use of the PMN substance.

Physical and Chemical Properties Worksheet (PMN page 13, Form page 18)	☑	Knowing specific physical chemical properties of the substance would allow competitors to derive information about the substance identity. With such information, the PMN substance could be recreated using common synthetic techniques which could compete in this marketplace, with or without patents.
Other information elements claimed as CBI	☑	Revealing such information would permit a competitor to specifically know that the submitter is seeking develop new products for the generic use disclosed in the PMN. This would result in harmful effects on submitter's competitive advantage.
Part III. Attachments.	**CBI Claim**	**Substantiation** *(*Explain how public disclosure of this information is likely to cause substantial harm to your business's competitive position.*)*
List of Attachments (Part III, PMN Page 12, form 17)	☑	Revealing such information would specifically reveal the number of supporting manufacturing process diagrams and the number of submitter-controlled operations. Revealing such information would permit a competitor to specifically know that the submitter is seeking develop new products for the generic use disclosed in the PMN. This would result in harmful effects on submitter's competitive advantage.
Health & safety studies or other test data (Note: TSCA section 14(b) places significant limits on what health and safety data may be withheld as CBI.)	☑	Only information that would reveal the substance identity or identity of the submitter are claimed CBI. All toxicity end points are disclosed in the public version of the health and safety studies.
SDS	☑	Information in the SDS would provide a link between the submitter identity and the new PMN substance. Revealing such information would permit a competitor to specifically know that the submitter is seeking develop new products for the generic use disclosed in the PMN. This would result in harmful effects on submitter's competitive advantage.
Physical Chemistry Property Worksheet	☑	Knowing specific physical chemical properties of the substance would allow competitors to derive information about the substance identity. With such information, the PMN substance could be recreated using common synthetic techniques which could compete in this marketplace, with or without patents.
Other:	☐	Click or tap here to enter text.

II. ADDITIONAL QUESTIONS

A. To the extent the submitter has disclosed information to others (both internally and externally), what precautions has the business taken? Please identify the measures or internal controls the business has taken to protect the information claimed as confidential.	
1. Non-disclosure agreement required prior to access.	☑ Yes ☐ No
2. Access is limited to individuals with a need-to-know.	☑ Yes ☐ No
3. Information is physically secured (e.g. locked in room or cabinet) or electronically secured (encrypted, password protected, etc.).	☑ Yes ☐ No
4. Other internal control measure(s). *(If yes please explain below.)*	☐ Yes ☐ No
Click or tap here to enter text.	
B. Does the information claimed as confidential appear in any public documents, including (but not limited to) safety data sheet, advertising or promotional material, professional or trade publication, or any other media or publications available to the general public?	☐ Yes ☑ No
Click or tap here to enter text.	
C. Does any of the information you are claiming as CBI contain (a) trade secret(s)[1]? *(If yes, please identify what information is being claimed as (a) trade secret(s).)*	☐ Yes ☑ No
Click or tap here to enter text.	
D. For what period do you assert the claim of confidentiality (please indicate between 1-10 years or until a certain event)?[2]	
10 Years	
E. Has EPA, another federal agency, or court made any confidentiality determination regarding information associated with this substance? *(If yes, please explain the outcome of that determination and provide a copy of the previous confidentiality determination or any other information that will assist in identifying the prior determination.)*	☐ Yes ☑ No
Click or tap here to enter text.	
F. (*Applicable only to SNUNs or to requests to modify a granted LVE or LOREX submission AND only if chemical identity is claimed as confidential.*) When this chemical substance leaves the site of manufacture in any form, *e.g.,* as product, effluent, emission, what measures are taken to guard against the discovery of its identity? When the chemical substance leaves the site in a product that is available to the public or your competitors, can the chemical substance be identified by analysis of the product?	
Click or tap here to enter text.	
Additional comments:	
Click or tap here to enter text.	

III. SUBSTANTIATION CERTIFICATION

Do you wish to claim this substantiation as CBI?

TSCA section 14(c) requires that persons asserting a CBI claim shall certify to the validity of the claims. By the marking of a yes, you are certifying to the truth of the below statements.

☑ Yes

☐ No

I hereby certify to the best of my knowledge and belief that all information entered on this form is complete and accurate.

I further certify that, pursuant to 15 U.S.C. § 2613(c), for all claims for confidentiality made with this submission, all information submitted to substantiate such claims is true and correct, and that it is true and correct that

(i) My company has taken reasonable measures to protect the confidentiality of the information;
(ii) I have determined that the information is not required to be disclosed or otherwise made available to the public under any other Federal law;
(iii) I have a reasonable basis to conclude that disclosure of the information is likely to cause substantial harm to the competitive position of my company; and
(iv) I have a reasonable basis to believe that the information is not readily discoverable through reverse engineering.

Any knowing and willful misrepresentation is subject to criminal penalty pursuant to 18 U.S.C. § 1001.

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[1] **"Trade secret"** is defined as "a secret, commercially valuable plan, formula, process, or device that is used for the making, preparing, compounding, or processing of trade commodities and that can be said to be the end product of either innovation or substantial effort." Public Citizen Health Research Group v. FDA, 704 F.2d 1280, 1288 (D.C. Cir. 1983).

[2] Information with withdrawn CBI claims may be made available to the public without further notice.